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Thank you

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Progress Towards Gene Therapy for Cancer

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This review highlights the current strategies being employed towards gene therapy of cancer. Conceptually, the most simple diseases to treat with gene therapy would be monogenic inherited diseases, such as hemophilia. However, the vast majority of current gene therapy trials are for treatment of cancer patients, due to the recognition of gene alterations in cancer and the critical need for improvement of cancer therapy. Gene-based therapies for cancer in clinical trials include strategies that involve immuno-therapy, induction of drug sensitivity in tumor cells or resistance to chemotherapy of critical host tissues, and compensation for oncosuppressor loss or ablation of oncogenes. Two broad approaches have been used to deliver DNA to cells, a series of viral vectors and the use of plasmid DNA vectors, which have different advantages with regard to efficiency of gene transfer, ease of production and safety.
Examined objectively, many of the first studies in cancer gene therapy clinical trials have provided information of critical importance for the design of more efficient second-generation protocols.
Gene therapy represents one of the most important developments in oncology, however, before this can be realized as standard treatment the technical problems of gene delivery and safety must be overcome. Here we focus on methods and strategies used to achieve cancer gene therapy and the current clinical trials.

Key Words: Gene Therapy, Cancer, Clinical Trials, Vectors, Immunotherapy

Modifying cells to express new proteins has long been envisioned as means of correcting human inborn genetic defects. Loosely applied, the term gene therapy can refer to any clinical use of recombinant DNA technology to modify somatic cells. Alternatively, the term can be applied more strictly so that it denotes only the replacement of defective or missing genes. The concept of gene therapy (1) follows logically from the observation that certain diseases are caused by the inheritance of a single functionally defective gene, diseases that are potentially treated by replacement with a normal functional copy of the gene. While the classic use of gene therapy is for correction of diseases caused by single-gene defects, gene transfer has been widely used over the past few years to develop novel therapies for cancer. In fact, the majority of the approved gene therapy protocols involve cancer patients. At this moment about 68% of current protocols in gene therapy are for treatment of different tumors (Tab.I). Human neoplasms are generally recognized to be the result of accumulated genetic lesions that culminate in the transformed phenotype. As the disease state results from defined genetic mutations, gene therapy techniques may provide a rational basis for intervention and this approach may offer a valid method to achieve anti-tumor effects (2).

The current strategies for gene therapy of cancer, which include compensation for mutations, molecular chemotherapy, and genetic stimulation of the immune response, have all undergone rapid development (3, 4) and are currently being tested in clinical trials (Fig.1). These strategies include *ex vivo* and *in vivo* cytokine gene transfer (5), prodrug sensitization, inactivation of oncogene expression, gene replacement of inactivated tumor suppressor genes and the use of drug resistance genes to protect the bone marrow from high-dose chemotherapy (6). Recent studies have suggested a new target in anti-tumor gene therapy, based on inhibi-

tion of tumor associated angiogenesis (7, 8, 9, 10).

Critical points in gene therapy development are the optimization of gene delivery and the assurance of safety for both the patient and the clinical personnel. Key studies are focused on improvement of viral vectors to reduce toxicity and immunogenicity, to enhance vector targeting and tissue-specificity, and to regulate gene expression (11, 12).

In this review we will focus on the present strategies in gene therapy in oncology, the current clinical trials in gene therapy of cancer and the principal vectors used.

Current Approaches in Tumor Gene Therapy

Immunotherapy

Increasing evidence that the immune system may be able to eradicate human tumors has led to interest in combining gene therapy with cellular immuno-therapy. Tumor regression may be induced by manipulating the human immune response with immune stimulating cytokines (13, 14) or expression of tumor antigens in engineered cells (15, 16). Many protocols are based on use of tumor-infiltrating lymphocytes (TILs), tumor cells or fibroblasts expressing cytokine genes. Expression of cytokine genes such as IL-2 in TIL cells was based on their capacity to penetrate the tumor mass (15). Currently the use of this approach is complicated by systemic toxicity. Another approach is to make tumor cells more immunogenic, since they are often defective in expression of class I or class II HLA molecules resulting in poor antigen presentation. Phase I/II trials of injection of HLA genes resulted in an immune-mediated partial regression of some tumors. (17). Recent clinical trials performed with a gene coding for HLA-B7 effectively showed anti-tumor (autologous) and anti-HLA-B7 (allogeneic) cytotoxic lymphocyte immune responses in those patients with regression of the tumor (18, 19, 20). Alternatively, direct in situ inoculation of DNA expressing accessory molecules such B7-1 or B7-2 may be used for effective induction of the immune response against tumor antigens (21, 22).

Drug Sensitivity Genes

Suicide genes, such as that encoding the herpes virus Thymidine Kinase (TK) gene, can be introduced directly in tumor cells to induce cell death. In presence of gancyclovir the TK gene product phosphorylates the pro-drug to a highly cytotoxic modified nucleotide that suppresses DNA synthesis in the transduced cells. The advantage of this approach consists in the capacity of the converted drug to transfer through gap junctions into non-transfected "bystander" cells (bystander effect), partially bypassing the problems linked to the transduction efficiency (23). Clinical trials based on this approach have been developed using either fibroblasts as vector cells (24) or intra lesional injection of a packaging cell line producing a retrovirus harboring the TK gene (25, 26).

The major difficulty with this approach is the need to selectively target tumor cells while avoiding transduction and killing of normal tissue. Due to this limitation, at present "suicide" gene therapy has been only applied to local cancer therapy, for instance brain tumors (26).

One of the most interesting applications of this approach has been to utilize the TK gene to control the eventual development of graft-vs-host disease (27). Heterologous bone marrow transplantation controls residual lymophomas better than autologous transplantation, however these graft cells can also react against the normal host tissues. To control the development of host reactive disease the bone marrow cells are first transduced with a vector carrying the TK gene (28); if graft-vs-host disease develops, glancyclovir can be used to control the reaction against host tissues.

Tumor Suppressor Genes

Tumor suppressor genes participate in critical cell functions including signal transduction, gene transcription, and control of cell proliferation or cell death (29, 30, 31). Loss or mutation of both alleles, and sometimes even a single allele, of these genes usually contributes to the malignant phenotype (30). Theoretically, substitution of mutated or deleted tumor suppressor gene with the functional copy could restore the original suppressor function. The p53 gene is one of the most studied tumor suppressor genes, since mutation of p53 is involved in a large spectrum of tumor types (32, 33, 34).

p53 mutations effectively contribute to uncontrolled cell growth, substantial data *in vivo* and *in vitro* show that introduction of a wild-type copy of the p53 gene can restore normal function and suppress malignancy (35, 36). Several clinical trials are being performed using this approach, in particular with adenoviral vector-p53 expression systems (37, 38). Administration of a retrovirus expressing a p53 gene to patients with non-metastatic lung cancer showed strong tumor regression at the end of the first month after treatment (39, 40). However, the theoretical necessity for transduction of

all the tumor cells remains a significant barrier for this approach although a "bystander" effect has been reported.

Oncogenes

Altering the malignant phenotype by blocking oncogenes is an attractive strategy that is being evaluated in several clinical trials. Many of these studies use an anti-sense approach based on stable expression of a gene construct that has a base sequence complementary to the RNA sequence of an activated oncogene (41). This approach requires high efficiency of transduction and expression of the transgene, this is one of its major limitations. Alternative methods for reduction or inhibition of activated oncogenes are based on transfer of ribozyme constructs (42), intracellular scFv antibodies (43) and oligonucleotide anti-sense transfection (41).

Gene Marking

Gene marking studies, using different types of vectors, have been used to demonstrate that contaminating gene-marked tumor cells can contribute to relapse following autologous stem cell transplants (44). Examples of this approach are studies of acute myeloblastic leukemia, chronic myeloid leukemia and neuroblastoma. The clinical applications of this approach in trials are aimed at better understanding whether human somatic transduction can be safe, whether the transduced genes could be durably expressed, and whether the transduction process would affect cell properties (45). This method is also supplying valuable information on the dynamics of repopulation following bone marrow transplantation and on the homing of transplanted cells. One trial started in 1987 (15) was performed by re-implanting autologous TILs marked with retroviral vectors expressing a NeoR gene under the control of MoMuLV LTR. This resulted in no detection of contaminating amphotropic viruses and reverse transcriptase activity in TIL samples, further, the transgene was also detected in tumor biopsies, demonstrating that *ex vivo* transduction did not abolish the natural tumor-targeting properties of the TIL.

In autologous bone marrow transplantation, *ex vivo* gene marking of normal and malignant hematopoietic cells allows the cells to be subsequently tracked *in vivo*. One current clinical trial is *ex vivo* transfection with a retroviral vector carrying the neo Resistence gene of autologous bone marrow cells of patients with either acute myeloid leukemia or neuroblastoma. (46). Double gene marking has also been performed (47) to mark either peripheral blood or bone marrow CD34+ cells.

Chemoresistance Genes

Another approach under investigation is the transfer of multi-drug resistance genes into normal cells to allow the use of higher doses of chemo-therapeutic drugs. Bone marrow protection during chemotherapy is one of the principal targets of many trials performed by transducing the multiple drug resistance gene in blood-derived stem cells or into normal bone marrow (48, 49). This permits more intensive chemotherapy and presumably a higher therapeutic ratio. Clinical trials are in progress where Multiple Drug Resistance gene transduced cells will be used to treat patients with high-dose Taxol or other drugs.

Gene Therapy for Brain Tumors

Gene therapy is particularly appealing for the treatment of tumors of the central nervous system (50). Brain tumors are difficult to treat by any conventional means. The high invasiveness reduces the success of surgical treatment while the presence of subpopulations of cells that temporarily withdraw from the cell cycle protects the tumor from the effects of chemotherapy. Drug treatments are also hindered by the reduced permeability of the blood brain barrier. In addition, the CNS is under reduced immune surveillance. Approaches that target dividing cells such as retroviral delivery or prodrug converting enzymes that produce replication disrupting drugs are particularly suited for gene therapy of brain tumors since they specifically target dividing neoplastic cells while sparing the non-dividing neurons. Eventually, transduction of dividing endothelial cells of the brain microvasculature can be therapeutic since brain tumors are highly dependent on neo-angiogenesis. However, replication-disrupting drugs such as nucleotide analogs reach bystander cells only via gap-junctions and are unlikely to reach all the cells of a tumor. Delivery of diffusible factors that induce terminal differentiation or apoptosis (51) or that repress neo-angiogenesis are more promising strategies. Immune-modulatory approaches, such as cytokine delivery to the CNS (52, 53) or the peripheral injection of *ex vivo* transduced tumor cells, which have proven effective in other diseases, must take into account that inflammation in the CNS may severely compromise normal brain cells.

Gene Delivery Systems

For gene therapy radically different technologies

have been developed to achieve the high efficiency gene transfer necessary to vector DNA into large numbers of cells *in vivo*. At present, only systems to constitutively express proteins have been used in trials, more elegant regulated systems for protein expression are being developed *in vivo* so that protein expression can be directed for instance the use of tissue specific promoters, for example the melanin-specific tyrosinase promoter for melanoma (54, 55).

Retroviral Vectors

Retroviruses are a class of enveloped viruses containing a single stranded RNA molecule that, after infection, is reverse transcribed into a double stranded DNA that integrates in to the host genome and is expressed as protein. The viral genome is approximately 10 Kb and contains three genes: Gag, for core proteins, Pol, for reverse transcriptase and Env, for viral envelope proteins. At the end of the genome are the Long Terminal Repeats that contain promoter region and the integration sequences, and the sequence PSI (Y) necessary for the packaging of the virus.

Most vectors are derived from the murine Moloney leukemia virus (56), which contains double-stranded RNAs that replicate through a DNA intermediate in host cells. Furthermore, the recombinant virus can utilize either an ecotropic envelope, which recognizes only mouse cells, or an amphotropic envelope able to recognize human and other cells. In this vector, the functional genes gag, pol, env, are removed and replaced by the transgene of interest (56). The resulting DNA is incorporated into the host genome during cell replication. The genetically altered retroviruses are produced by cells that have been modified to carry the viral polymerase, the envelope gene and the packaging sequence in separate inserts (56, 57). As these vectors require cell replication for complete infection and integration, their ability to transduce is restricted to replicating cells. The integrated provirus remains present for the lifetime of the cells and is transferred to progeny cells.

The requirement for an active state of proliferation can be viewed as a limitation, but in some cases this could be a clear advantage if selective expression of transgene is to be achieved in actively proliferating tumor cells. Furthermore, extensive evidence shows that *in vivo* tumor cells are preferentially targeted by retroviruses (39).

The use of retroviruses in gene therapy, however, shows many limitations. These are linked to their limited capacity of about 8Kb of insert DNA, to the current achievable viral titers (about 10^7), low compared to that needed for treatment of large tumors, and for the risk of generation of recombinant replication competent viruses. Although the insertion of the viruses into the genome is an advantage, the potential for insertional mutation remains a concern. Finally, retrovirus are inactivated by C1 complement protein (58) and anti-alpha galactosyl epitope antibodies, both present in human sera. However, at this moment retroviral vectors are the most commonly used in all current clinical trials (Fig.2).

Adenoviral Vectors

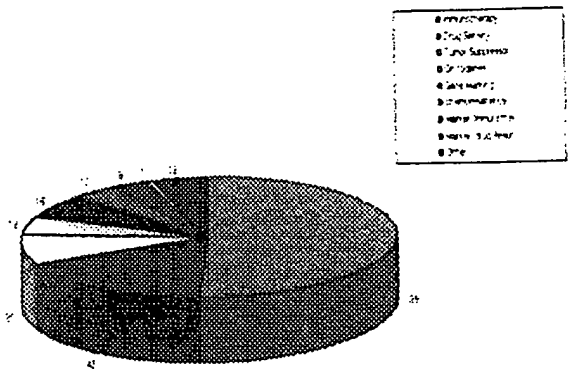
Adenoviruses are non-enveloped viruses containing a linear double stranded DNA genome. Their life cycle does not involve integration into the host genome, rather they replicate as episomal elements in the nucleus of the host cells. This property excludes the insertional problems linked to retroviral vectors.

Viral vectors based on the Ad5 virus are double-stranded DNA viruses capable of high levels of transduction. The wild type adenovirus genome is about 35 Kb of which about 30 Kb can be replaced with transgene(s). The 1st generation Ad vectors are replication-incompetent due to the ablation of the E1 viral sequence, replaced with transgenes of about 5-8 Kb. Vectors are produced in a packaging cell line expressing the sequences for viral replication. Other Adenoviral vectors were subsequently produced with elimination of the E2 and E3 sequences (respectively 2nd and 3nd generation) allowing insertion of larger transgenes and, above all, reducing the host immune response with an increase in the duration of therapeutic gene expression (59).

Until recently, the mechanism by which adenovirus targets cells was poorly understood. One study (60) showed that following intravenous injection, 90% of the vectors were degraded in the liver by a non-immune-mediated mechanism.

Although adenoviruses have been extensively used in gene therapy, these vectors are also not without drawbacks. Adenoviruses have 47 serotypes and their large diffusion in humans often results in a strong immune response in the host due to presence of viral proteins (61), sometimes even higher in case of previous exposure to the virus. In fact, severe reactions and even death are now being reported when using high doses of these vectors (62, 63). Furthermore, unlike retroviral vectors, Adenoviral vectors can transduce replicating and non-replicating cells with a high level of infection, but they do not integrate into host cell DNA, so that the duration of expression is very limited, in contrast to retroviral-mediated gene transfer.

CURRENT APPROACHES IN TUMOR GENE THERAPY



Type of trial	NUMBER	%
Immunotherapy	128	50,8
Drug Sensitivity	45	17,8
Tumor Suppressor	20	7,9
Oncogenes	12	4,8
Gene Marking	16	6,3
Chemoresistence	10	4
Marker/Immunother.	8	3
Marker/drug Resist.	1	0,4
Other	12	4,7
TOTAL	252	100

Fig.1 - The clinical trials different in gene therapy of cancer in the world the under study. The immunotherapy approach represents about 50% of approved trials

Adeno-Associated Vectors

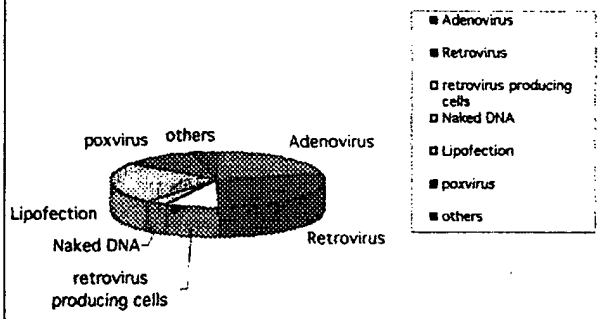
The Adeno-Associated Virus (AAV) is a member of the single stranded DNA parvovirus family with a small genome (about 4.7Kb). AAV vectors have, as predominant features, the complete absence of any association with human disease, the ability to infect a wide range of cells and a site specificity of integration (64, 65). The wild type genome consists of two genes, rep and cap, coding for viral replication, gene expression and integration into the host genome. At either end of the viral DNA is a sequence named the Terminal Repeat that contains a promoter and regulatory sequences.

Production of recombinant vectors requires, in addition to the rep and cap genes, also helper adenovirus (AV) gene products. The conventional method to produce AAV vectors has been co-trasfection of two plasmids: one for the transgene and one for structural genes cap/rep, into adenovirus infected cells. This procedure was prone to contamination with adenovirus and wild type AAV. The most recent protocols remove all adenoviral sequences and rep resistance co-trasfection, greatly reducing these complications. In the absence of the rep gene, the drawbacks of AAV are primarily the limited size of the DNA that can be inserted and the loss of site-specific integration when the viral genes are removed (66). The wild type virus integrates at a specific site in chromosome 19 (65, 66) but this activity appears to be lost in the recombinant vectors.

Lentiviral Vectors

The outbreak of the AIDS epidemic and the subsequent intense study of HIV has also led to the development of vectors based on the lentivirus HIV-1. Lentiviruses are a subclass of retroviruses which are able to infect either proliferating or non proliferating cells and allow high levels of expression of a transgene. They encode for six more proteins as compared to the retrovirus derived from Moloney, (tat, rev vpr, vpu, nef and vif). The packaging cell lines that have been developed to produce these viruses have separate plasmids for a pseudotype of the env gene and the packaging construct supplying the structural and regulatory genes (67).

VECTOR USED IN CURRENT TRIALS OF CANCER



Type of vector	number of trials	%
Adenovirus	52	21,6
Retrovirus	68	28,2
Retrovirus producing cells	20	8,3
Naked DNA	8	3,3
Lipofection	58	24,1
Poxvirus	26	10,8
others	9	3,7
total	241	100

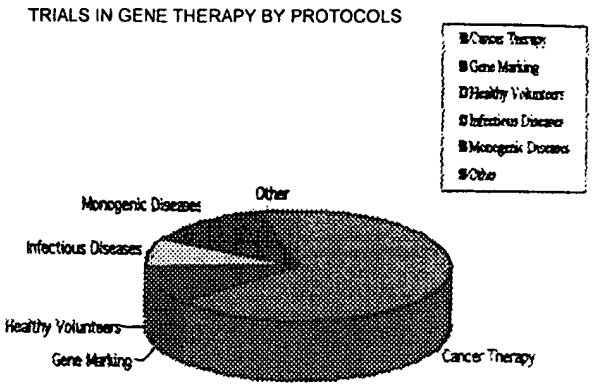
Fig.. 2 - Different vectors used in approved clinical trials. Retroviral vectors derived from Moloney Leukaemia virus are at this moment the principal vectors used in gene therapy trials. Lipofection is also an alternative method that is preferred to other viral vectors for safety and ease of manipulation

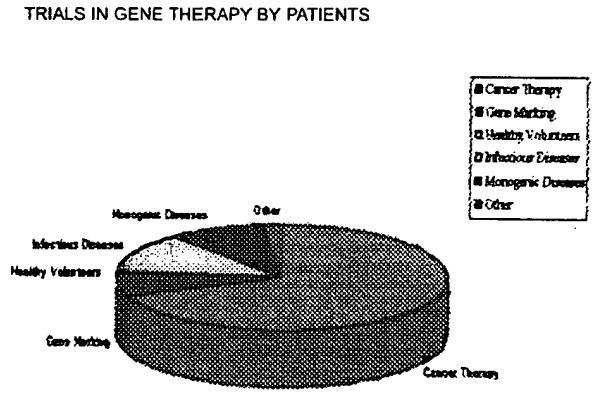
Tab I - Approved trials by disease. While the gene therapy approach was conceived for monogenic diseases, the major part of studies and clinical trials are for cancer therapy both in number of protocols and in number of patients.

CATEGORY	PROTOCOLS		PATIENTS	
	NUMBER	%	NUMBER	%
Cancer Therapy	252	63,6	2269	69,2
Gene Marking	41	10,4	227	6,9
Healthy Volunteers	2	0,5	6	0,2
Infectious Diseases	33	8,3	412	12,6
Monogenic Diseases	53	13,4	298	9,1
Other	15	3,8 66	2	
Total	396	100	3278	100

SPECIFIC TREATED CANCERS

Gynecological tumors (breast, ovary, cervix)
Nervous system tumors (glioblastoma, leptomeningeal carcinomatosis, glioma , astrocytoma, neuroblastoma)
Gastrointestinal tumors (colon, colorectal, liver metastases, post-hepatitis liver cancer)
Genito-urinary tumors (prostate, renal carcinoma)
Skin tumors (melanoma)
Head and neck tumors
Lung tumors (adenocarcinoma, small cell cancer, non-small cell cancer)
Mesothelioma
Hematological malignancies (leukemias, lymphomas, GvHD risk after alllogeneic BMT in leukemia, multiple myeloma)
Sarcomas
Germ cell cancers





These vectors are constructed, for example, using the core of HIV-1 and the surface protein from stomatite vesicular virus. Lentiviral vectors allow introduction of genes in practically all cell types with high levels of infection. Preclinical results with gene marking have been promising, showing prolonged *in vivo* expression in muscle, liver and neuronal tissue. (68, 69, 70)

The principal obstacles for the use of these vectors are the safety problems for clinical staff and patients, and the risk linked to potential generation of recombinant viruses as devastating, or even more so, than the parental HIV. Ablation of nearly all viral sequences, while maintaining the principal characteristics, is the current principal target of investigators in this field.

Herpes Simplex, Poxvirus, Vaccinia Virus Derived Vectors

The herpes simplex virus is a large double stranded DNA that is used above all for gene transfer into neuronal cells (71). The potential of producing latent infections, and the necessity to work with large viral DNA, are the principal problems for the use of these vectors. They also show potential immunological complications such as induction of chronic inflammation (72, 73, 74). Poxvirus vectors also are used in some clinical trials due to their capability to infect and express recombinant proteins in human cells without viral replication. For this characteristic they are often used for DNA vaccination trials.

Non Viral Vectors

Naked DNA

Viral vectors are only one approach to the genetic

Tab. II - Principal features of vectors for gene delivery into cells

vector	integration	Cell replication Required	Toxicity Immuno-genity	Size Insert	Targeting
Retrovirus	Yes	Yes	No	9- 12 Kb	No
Adenovirus	No	No	Yes	4-5 Kb	No
AAV	?	No	Yes	5 Kb	No
Lentivirus	Yes	No	Yes		No
Herpes virus	No	No	Yes	40-50 Kb	No
Poxvirus		No	Yes	>4Kb	No
Naked DNA	No	No	No	No limits	No
Liposomes	No	No	No	No limits	No

modification of target cells. The use of naked DNA may bypass many of the safety problems linked to viral vectors, this strategy also provides simple preparation and large-scale production. The low efficiency of *in vivo* transfection and expression may be the most limiting factor, but several studies are addressing optimization of delivery (75). Liposomes are lipid bilayers that contain a cationic surface able to entrap DNA charge interactions and to bind to the cellular membrane. Encapsulating DNA with liposomes or lipid carriers can facilitate cellular uptake and virtually 90% of all cell types are transfectable with this procedure (76). However, cell specificity targeting and distribution beyond the site of administration remain problems that are not yet solved for this approach.

Current Clinical Applications

Currently there are numerous clinical trials now in phase I and phase II study. The results available to date are in large part from phase I trials; with all major gene transfer approaches there is a high heterogeneity of cancer types being investigated (Table I). Phase I human trials are primarily designed to demonstrate safety and efficacy of this approach and will not provide complete results. The patients recruited have been principally those with advanced metastatic cancer and the early results are being comparable to single chemotherapic agents.

In human gene therapy of cancer, at this moment, the majority of approved trials are based on immunotherapy to increase a pre-existing immune-response, followed by drug sensitivity gene studies. The results available are not definitive and more investigation is necessary to optimize the gene therapy of cancer. However, all the phase I trials have been conducted on low patient numbers who were not responsive to conventional therapy. Even if only sporadic cases show a complete regression, this, coupled with the generally low toxicity of treatment, is an indication that gene therapy will eventually represent a possible alternative to the conventional therapies in use. Gene therapy could result in an improvement of quality of life of the patients as compared to the cytotoxic therapies now used. The morbidity associated with cancer gene therapy is significantly lower with respect to conventional treatments, however a more aggressive approach may also be beneficial. The most used vectors are the retroviral vectors that give, in most cases, a more efficient gene transfer into cancer cells than into normal cells. Alternatively, many approved trials are based on lipofection that even though they show low delivery of transgene, allow easy, large scale production of therapeutic DNA without the safety problems linked to viral manipulation (Fig. 2 - Tab. II).

General Problems Associated with Cancer Gene Therapy

Tumors are composed of large numbers of cells that have escaped from growth control. Re-establishing growth control or cell killing will significantly influence tumor growth only if the therapeutic agent reaches most or all transformed cells. This is a major limitation for many tumor therapies, especially when solid tumors are considered.

Gene therapy makes no exception to this rule. Penetrance throughout the tumor is difficult to achieve with viral treatments and viral transduction efficiency is an additional limitation. Therefore, gene therapy strategies that provoke bystander effects (i.e. prodrug activating genes) appear more promising than approaches where the anti-tumoral effect is limited to the cells actually transduced. Production of diffusible factors, such as cytokines, appears even more attractive since they have the potential to reach the entire tumor. Adoptive immunotherapy of tumors modifies tumor cells *ex vivo* in order to stimulate the immune system against all the tumor cells, including those that do not express the gene therapy construct. In this case, gene therapy could reach even undetected distant metastases.

Safety Aspects

Safety of viral vectors is, at present, one of the most critical aspects of human gene therapy. The recent treatment related death of a young man due to a systemic inflammatory response to the therapeutic aden-

oviral construct has risen major concern on the safety issue (62, 63). The Recombinant DNA Advisory Committee (RAC), in examining this event, presented a list of problems linked to adenovirus safety. Standards for adenovirus titration and the measurement of transgene expression and a better assessment of immune status before and after treatment are needed. The Committee also indicates that studies of vector biodistribution must be performed and a better quality control for the integrity of vector DNA is to be obtained (62).

Conclusions

The heterogeneity of tumors makes it unlikely that a single principal approach will prove effective for all tumors. Future approaches probably will rely on combinations of immune adoptive therapy, molecular chemotherapy and/or prodrug conversion eventually in addition to protective treatments of normal cells (drug resistance). The current development of gene therapy vectors considers target cell specificity, persistance and level of transgene expression, cell-type specific expression and reduction of immunogenicity of the vectors.

In spite of the controversial opinions on gene therapy, both in the public and in the scientific community, this approach probably provides a valid contribution to future treatments of the cancer patient. Certainly, much must be done before this approach comes to the clinical routine.

Acknowledgements: These studies were supported by grants from the Fondazione Cassa di Risparmio di Genova e Imperia, the MURST to L.S. and the AIRC to D. N. C.M. is a FIRC fellow.

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Received: July 1, 2000

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An Update on Angiogenesis Therapy

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ABSTRACT: Angiogenesis is a novel approach for the therapy of various ischemia-related pathophysiologic conditions. Proangiogenic growth factors have shown promising results in preclinical studies using protein- and gene-based therapies. However, their success in clinical trials is hindered by the lack of an optimal delivery strategy that would provide sustained and localized levels of the growth factors in the diseased tissue. Targeted delivery of proangiogenic agents is expected to demonstrate therapeutic efficacy of growth factors at relatively lower doses, without the risk of systemic toxicity in terms of unwanted angiogenesis. To achieve the above objectives, various drug delivery systems are under investigation. This review describes the basic mechanism of action of growth factors, their current status in preclinical and clinical studies, and the issue of drug delivery.

KEY WORDS: growth factors, ischemia, drug delivery, gene therapy

I. INTRODUCTION

Approximately 15 million patients suffer from coronary and peripheral atherosclerotic diseases in the United States alone *(National Heart, Lung, Blood Institute Fact Book,* 1997*)*. Ischemia of the heart remains the leading cause of morbidity and mortality in the world (Murray and Lopez, 1997). Despite development of medical and surgical therapies, many of these patients remain at risk for myocardial infarction, limb loss, and death. Recent investigations have revealed that recombinant formulations of proangiogenic agents could be used to augment development of collateral blood vessels. This novel strategy for the treatment of vascular insufficiency, also known as therapeutic angiogenesis, is at the forefront of research for the therapy of various ischemia-related pathophysiologic conditions. Angiogenesis, the sprouting of new blood vessels from preexisting vasculature (Folkman, 1995a), is essential for the proper development of the vascular system. Besides its role in physiological situations, including wound healing, ovulation, and placental growth, angiogenesis has been implicated in several pathological conditions, such as retinopathies, rheumatoid arthritis, vascular diseases, psoriasis, and tumor growth (Folkman and Klagsbrun, 1987; Folkman, 1995; Polverini, 1995; Folkman and D'Amore, 1996). Angiogenesis is a natural biological response of a tissue to hypoxia or ischemia and has been shown to occur because of the release of endogenous growth factors (Kumar et al., 1983; McNeil et al., 1989). However, this compensatory response to the hypoxic stimulus is insufficient in magnitude to return perfusion levels to normal (Baffour et al., 1992; Rosengart et al., 1997). Furthermore, chronic hypoxia results in a reduction in the ability of cells to produce growth factors in response to future episodes of hypoxia, and this is responsible, at least in part, for the inadequate compensatory angiogenesis observed in several chronic ischemic disorders (Levy, 1999). Therefore, the rationale behind therapeutic angiogenesis is that supplementing the deficient endogenous growth factor response with external growth factors would induce neovascularization adequate to reconstitute the ischemic tissue to its state of normal perfusion. This review focuses on the basic mechanism of action of proangiogenic growth factors, describes the current status of preclinical and clinical findings, and addresses the issue of delivery strategies for proangiogenic agents.

1045-4403/01/$5.00

II. VASCULOGENESIS AND ANGIOGENESIS IN THE DEVELOPMENT OF THE VASCULAR SYSTEM

The adult vascular endothelium differs from embryonic vascular endothelium in that the adult endothelium is mature, has a low turnover, and displays organ-specific characteristics (Risau, 1995). During embryogenesis, endothelial progenitor cells, or angioblasts, differentiate in situ into early endothelial cells that arrange themselves into a primary capillary network through a process called "vasculogenesis" (Carmeliet and Collen, 1999). The development and remodeling of this primary vasculature into a complex, organized network involves "angiogenesis" (Risau, 1997). Angiogenesis can occur by three mechanisms, namely, the sprouting of enlarged capillaries, intussusception or splitting of capillaries by pillars of periendothelial cells, or by the formation of transendothelial cell bridges within the vessel, which subsequently divide into individual capillaries (Carmeliet, 2000). The process of angiogenesis involves a complex sequence of events (Figure 1) beginning with vasodilation, which is mediated by endothelial nitric oxide production (Ziche et al., 1997). Proteolytic enzymes (chiefly plasminogen activator) secreted by the vascular endothelium cause the degradation of the surrounding basement membrane (Zimmerman et al., 1999). Subsequently, endothelial cells from adjacent established vessels begin to migrate toward the angiogenic stimulus and proliferate, arranging themselves into tubular structures. Accumulation of extravascular fibrin, as a consequence of vascular hyperpermeabil-

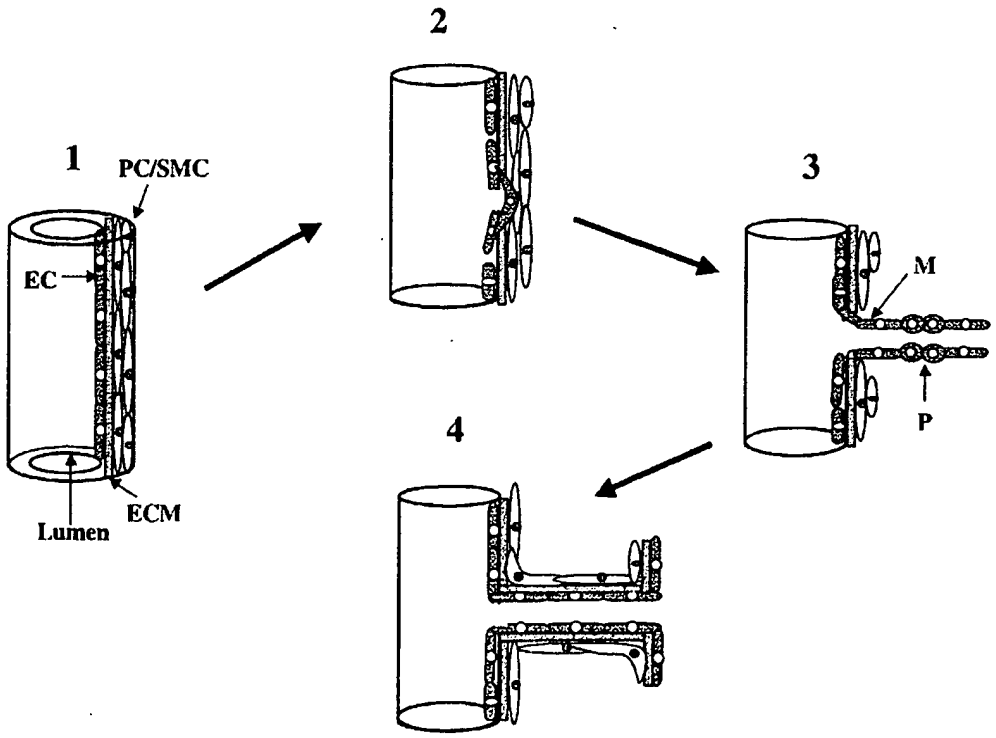

FIGURE 1. Schematic representation of mechanism of angiogenesis. (1) Blood vessels consist of endothelial cells (EC) on the luminal side followed by the extracellular matrix (ECM), basement membrane, and pericytes or smooth muscle cells (PC/SMC). (2) Angiogenesis begins with vasodilation and the activation of EC, followed by the degradation of ECM and basement membrane dissolution. (3) EC begin to migrate (M) toward the angiogenic stimulus and proliferate (P) causing elongation of the sprout. (4) New lumen formation is accompanied by regeneration of ECM and basement membrane and SMC recruitment.

ity, is an important step in the process of angiogenesis (Dvorak et al., 1995). The fibrin forms a provisional extravascular matrix that regulates the migration of endothelial cells and fibroblasts. Pericytes are specialized cells that transform the mature endothelium into a quiescent, nonproliferative state (Denekamp and Hill, 1991). At the end, angiogenesis involves an influx of pericytes that envelop the newly formed microvasculature (Zimmerman et al., 1999).

III. ROLE OF PROANGIOGENIC GROWTH FACTORS IN ANGIOGENESIS

Several growth factors, such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF-β), fibroblast growth factors (FGFs), and vascular endothelial growth factor (VEGF), are potent angiogenic agents (Table 1). These factors regulate angiogenesis—either directly by their action on endothelial cells or indirectly by activating surrounding cells to produce other factors that regulate angiogenesis, or by the modulation of receptor activities (Yoshida et al., 1997). Among factors that promote neovascularization, vascular endothelial growth factor (VEGF) and fibroblast growth factors (aFGF and bFGF) have been studied extensively (Table 2). VEGF and FGF positively regulate many endothelial cell functions, including proliferation, migration, and tube formation in vitro, leading to the notion that these factors are direct-acting proangiogenic

TABLE 1
Endogenous-Positive Regulators of Angiogenesis

Proangiogenic agent	Mechanism of angiogenic action
Vascular permeability factor/vascular endothelial growth factor (VPF/VEGF)	Promotes EC survival, proliferation, and migration; increases vascular permeability; induces production of urokinase plasminogen activator (Dvorak et al., 1995; Ferrara, 1999a)
Fibroblast growth factors (FGFs)—acidic and basic	Stimulate EC to proliferate and migrate, increase protease production, and undergo morphogenesis (Folkman and Klagsbrun, 1987)
Epidermal growth factor (EGF)	Stimulates EC proliferation; increases VEGF secretion in glial cells (Valter et al., 1999)
Angiogenin	Activates proteases, mediates cell adhesion, and promotes EC proliferation and tube formation (Strydom, 1998)
Transforming growth factor (TGF)-β	Induces angiogenesis by stimulating production of direct-acting positive regulators (Pepper, 1997a). Inhibits EC proliferation and migration in vitro. Potential mediator of the resolution phase of angiogenesis. (Pepper, 1997b)
Platelet-derived growth factor (PDGF)	Induces VEGF expression in EC (Wang et al., 1999)
Tumor necrosis factor (TNF)-α	Indirect positive regulation by stimulating production of proangiogenic molecules, such as VEGF, bFGF, and IL-8 (Yoshida et al., 1997)
Platelet-derived endothelial cell growth factor (PD-ECGF)	Stimulates EC DNA synthesis and chemotaxis; induces production of FGF (Ishikawa et al., 1989)
Placenta growth factor (PlGF)	Interacts with VEGF to modulate its mitogenic, chemotactic, and vascular permeability-inducing properties (Carmeliet and Collen, 1999)
Interleukin (IL)-8	Induces EC tube formation (Shono et al., 1996)
Hepatocyte growth factor (HGF)	Promotes EC migration, invasion, and production of proteases (Bussolino et al., 1992; Rosen et al., 1993)
Proliferin	Induces EC migration; may regulate the initiation of placental neovascularization (Jackson et al., 1994)
Angiopoietin-1	Expressed in close proximity to developing blood vessels (Davis et al., 1996); involved in the later stages of angiogenesis; aids vessel maturation and stabilization (Davis and Yancopoulos, 1999)

TABLE 2
Preclinical Studies Using Recombinant Proangiogenic Factors for Therapeutic Angiogenesis

Angiogenic factor	Dose	Mode of administration	Animal model	Results and conclusions	Refs.
bFGF	1 μg/day 3 μg/day	IM (daily for 2 weeks)	Rabbit hind limb ischemia	Dose-dependent increase in collateral vessel development in ischemic tissue	Baffour et al., 1992
rh-bFGF	10 μg	IC	Dog myocardial infarct	Increase in number of arterioles and capillaries in infarct	Yanagisawa-Miwa et al., 1992
bFGF	0.12 μg/kg body weight	IC via slow-release beads	Swine model of myocardial infarction	Enhanced myocardial neovascularization within 2 weeks	Battler et al., 1993
$rhVEGF_{165}$	0.5 mg 1 mg	Single IA bolus	Rabbit hind limb ischemia	Dose-dependent increase in collateral vessel development; evidence for the concept that angiogenic activity of VEGF is sufficient to provide therapeutic benefit	Takeshita et al., 1994a
$rhVEGF_{165}$	0.2, 0.5, or 1 mg/day	IM (daily for 10 days)	Rabbit hind limb ischemia	Dose-dependent augmentation in limb perfusion and increased collateral formation; support for the hypothesis of therapeutic angiogenesis	Takeshita et al., 1994b
$rhVEGF_{165}$	45 μg daily	IC (28 days via catheter)	Dog model of coronary insufficiency	Enhanced development of small coronary arteries supplying ischemic myocardium, resulting in marked augmentation of collateral blood flow and suggesting a new therapeutic approach for the treatment of myocardial ischemia	Banai et al., 1994
bFGF	4–5 μg	Periadventitial via heparin-alginate beads	Chronic porcine myocardial ischemia	Improvement of coronary flow, reduction in infarct size, and prevention of pacing-induced hemodynamic deterioration	Harada et al., 1994
bFGF	110 μg	Daily bolus injection in the ischemic myocardial zone for 28 days	Canine myocardial ischemia	bFGF has acute effects as a coronary vasodilator and enhances maximal collateral blood flow	Unger et al., 1994
$rhVEGF_{165}$	1 mg 5 mg	Single IV bolus	Rabbit hind limb ischemia	Single-bolus systemic administration of VEGF enhances collateral vessel formation in the ischemic limb and may be a feasible therapeutic strategy for patients with lower-extremity ischemia	Bauters et al., 1995

rhVEGF + rh-bFGF	VEGF(500 μg) + bFGF (10 μg)	Single IA bolus via infusion catheter	Rabbit hind limb ischemia	Synergism between two angiogenic agents with different target cell specificities in the augmentation of collateral circulation	Asahara et al., 1995
$rhVEGF_{165}$	2 mg	IC bolus	Chronic porcine myocardial ischemia	Augmented flow to collateral-dependent ischemic myocardium, but half the VEGF-treated animals died because of severe hypotension following VEGF administration	Hariawala et al., 1996
VEGF	2 μg	Extraluminal administration to LCX myocardium	Chronic porcine myocardial ischemia	Improvement of coronary flow and preservation of endothelium-dependent microvessel relaxation	Harada et al., 1996
Heparin aFGF aFGF/heparin	50 μg 1 μg aFGF (1 μg) + heparin (50 μg)	SC (daily for 10 days)	Rat hind limb ischemia	Angiogenesis is significantly accelerated by the administration of heparin alone and is accelerated to a greater extent by the administration of aFGF with or without heparin	Rosengart et al., 1997
bFGF	100 μg/kg	IV, left atrial, Swan Ganz catheter, IC, or pericardial route	Mongrel dogs	Myocardial and systemic distribution depends on route of administration. Serum half-life of bFGF was 50 min. IC, left atrial, and pericardial delivery could be effective for myocardial angiogenesis, but bolus intravenous and Swan Ganz administration is ineffective	Lazarous et al., 1997
rh-bFGF	0.3 μg/day 1 μg/day	IM (daily for 2 weeks)	Ischemic limb of diabetic rats	bFGF enhances angiogenesis and, possibly, collateral circulation in ischemic limbs in a background of diabetes	Stark et al., 1998
$rhVEGF_{165}$	0.22 μg/kg/day	IA via 28-day osmotic pump	Rabbit hind limb ischemia	Enhanced neovascularization by controlled release of microgram quantities of VEGF	Hopkins et al., 1998
rhVEGF-C	500 μg	Single IA bolus	Rabbit hind limb ischemia	VEGF-C enhances angiogenesis in ischemic settings and may represent an alternative to VEGF-A for therapeutic angiogenesis	Witzenbichler et al., 1998
rh-HGF	500 μg each 3 mg/day	Local IA (twice) IV (5 days)	Rabbit hind limb ischemia	Decrease in vascular HGF might be related to the pathogenesis of peripheral arterial disease. Administration of rhHGF as potential cytokine supplement therapy for peripheral arterial disease	Morishita et al., 1999
bFGF	^{125}I-bFGF + bFGF (30 μg) + heparin (3 mg)	IC and IV	Normal and ischemic pigs	Low cardiac deposition with both modes of delivery.Three- to fourfold lower myocardial deposition by IV delivery as compared with IC administration. Tissue deposition dropped by 5- to 20-fold in 24 hours	Laham et al., 1999a

Note: IA, intraarterial; IC, intracoronary; IV, intravenous; SC, subcutaneous; IM, intramuscular; rh, recombinant human.

agents (Gospodarowicz, 1989; Ferrara et al., 1992; Dvorak et al., 1995). In contrast, TGF-α and TNF-α inhibit endothelial cell growth in vitro and can, therefore, be termed direct-acting negative regulators; however, they induce angiogenesis in vivo. It has been shown that these factors promote angiogenesis by stimulating the production of direct-acting positive regulators from stromal and inflammatory cells and are, therefore, considered indirect proangiogenic agents (Klagsbrun and D'Amore, 1991; Pepper et al., 1996).

A. Vascular Endothelial Growth Factor

Among all the proangiogenic growth factors discovered, VEGF has been extensively investigated for the purpose of therapeutic angiogenesis. The protein was initially isolated from guinea pig tumors, and it increased microvascular permeability when injected into guinea pig skin. It was therefore named vascular permeability factor (VPF) (Senger et al., 1983). A few years later, two groups independently reported the purification of a mitogen specific for endothelial cells, which they named vascular endothelial growth factor (VEGF) (Ferrara and Henzel, 1989) and vasculotropin (Plouet et al., 1989). It was subsequently discovered that VEGF is a potent, diffusible protein that is specifically mitogenic for vascular endothelial cells derived from arteries, veins, and lymphatics, and that the activities of VPF (Keck et al., 1989) and VEGF (Leung et al., 1989) are embodied by the same molecule. This finding led to the hypothesis that VEGF could play a pivotal role in the regulation of blood vessel growth to promote revascularization in conditions of insufficient tissue perfusion (Ferrara and Henzel, 1989; Leung et al., 1989).

VEGF exists as four different molecular species, namely, $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$, having 121, 165, 189, and 206 amino acids, respectively (Ferrara et al., 1992). The existence of a fifth isoform, with 145 amino acids, has also been reported (Poltorak et al., 1997). Alternative splicing of a single VEGF gene results in the generation of the different isoforms (Neufeld et al., 1996). $VEGF_{165}$ is the major species produced by various normal and transformed cells. It is a basic, heparin-binding, homodimeric glycoprotein with a molecular weight of ~45 kDa (Ferrara and Henzel, 1989). $VEGF_{165}$ has two high-affinity binding sites, flt-1 (*fms*-like tyrosine kinase) (de Vries et al., 1992) or VEGFR-1 and KDR (kinase domain region) (Terman et al., 1992) or VEGFR-2, which are expressed predominantly, if not exclusively, on vascular endothelium. VEGF acts directly and selectively on endothelial cells via these two transmembrane receptor tyrosine kinases (Figure 2). Soker et al. (1998) have reported the existence of a third receptor that binds $VEGF_{165}$ and is identical to human neuropilin-1. Although neuropilin-1 is not directly involved in signaling, it has been proposed that it enhances the effectiveness of KDR signal transduction. The complete signal transduction pathway downstream of the VEGF receptors is still unknown. Several studies have indicated that the two receptors have different signal transduction properties (Waltenberger et al., 1994; Seetharam et al., 1995). Binding of VEGF to the receptors leads to their dimerization and activation, followed by the recruitment of signal transduction molecules, including protein kinase C and members of the Ras pathway (Shibuya et al., 1999). VEGF has been shown to induce the phosphorylation of at least 11 different proteins in bovine aortic cells (Guo et al., 1995). Initiation of signal transduction downstream of the receptors leads to endothelial cell morphogenesis, monocyte migration, and tissue factor production (via flt-1) as well as endothelial cell differentiation, proliferation, and migration (via KDR) (Shibuya et al., 1999). VEGF is not only a mitogen but also an important survival factor for endothelial cells (Gerber et al., 1998; Ferrara, 1999a).

VEGF, also known as VEGF-A, is closely related to the placenta growth factor (PlGF), and both are distantly related in structure to the platelet-derived growth factors (PDGFs) A and B (Heldin et al., 1993). More recently, other polypeptides with structural similarities to VEGF, PDGFs, and PlGF have been identified. The VEGF family of growth factors now includes VEGF-B (Olofsson et al., 1996), VEGF-C (Joukov et al., 1996), VEGF-D (Yamada et al., 1997), and VEGF-E (Ogawa et al., 1998).

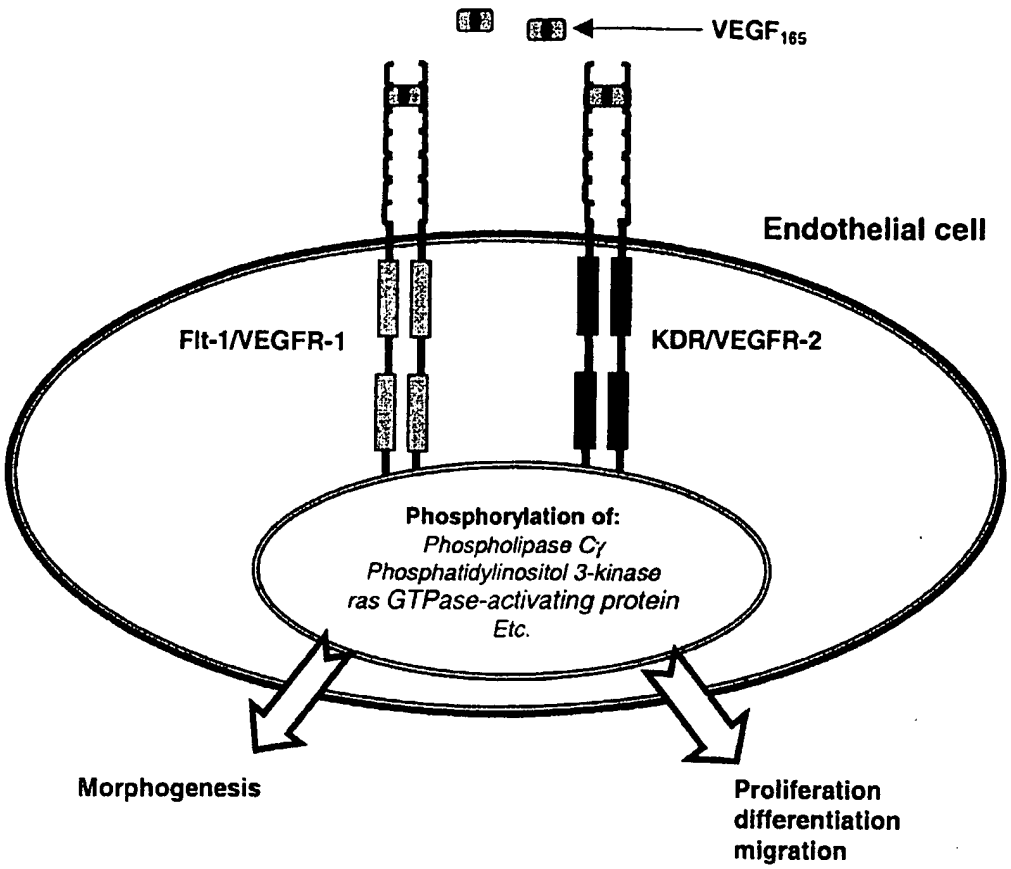

FIGURE 2. VEGF receptors and signal transduction.

B. Fibroblast Growth Factors

Fibroblast growth factors (FGFs) were initially discovered as mitogens for fibroblasts, but their activities are far more wide ranging. FGFs are a family of proteins that play vital roles in normal development, tissue maintenance, and wound healing (Galzie et al., 1997). They are potent regulators of cell proliferation, differentiation, and function. The FGF family presently consists of at least 9 members designated FGF-1 through FGF-9 (Galzie et al., 1997).

FGF-2, also known as basic FGF (bFGF), was first purified as a tumor-derived growth factor that stimulated the proliferation of capillary endothelial cells (Shing et al., 1984). bFGF is a cationic polypeptide that is synthesized as both an 18-kDa form and as higher molecular weight (22 to 25-kDa) forms (Pepper et al., 1996). It lacks a signal sequence that is necessary for the normal secretory pathway, and therefore remains mostly intracellular. However, receptors for FGF are present on cell surfaces, suggesting the existence of an export mechanism (Galzie et al., 1997). Unlike VEGF, the stimulatory effects of bFGF on proliferation are not restricted to endothelial cells, but include fibroblasts, astrocytes, neuroblasts, keratinocytes, osteoblasts, and smooth muscle cells (Burgess and Maciag, 1989; Klagsbrun, 1989). Acidic (FGF-1) and basic FGF were also found to induce the production of factors involved in the breakdown of the basement membrane and the migration of capillary endothelial cells into collagen matrices to form capillary-like tubes (Gospodarowicz et al., 1987), thus suggesting their role in the modulation of

angiogenesis (Burgess and Maciag, 1989; Klagsbrun, 1989).

C. Role of VEGF in the Regulation of Angiogenesis

VEGF increases vascular permeability (Roberts and Palade, 1995), which results in the leakage of plasma proteins, such as fibronectin and fibrinogen, into the perivascular compartment. This leads to activation of the extrinsic coagulation cascade such that a fibrin seal is formed that, along with other plasma proteins, provides a scaffold for migrating endothelial cells (Senger, 1996). VEGF may also induce vasodilation of arterioles via release of endothelial nitric oxide (Hariawala et al., 1996), resulting in increased blood flow through the tissue undergoing neovascularization. In addition, it may stimulate the migration of smooth muscle cells (SMCs) (Grosskreutz et al., 1999) and thus aid in the maturation of the newly formed endothelial tubes.

There is now increasing evidence that VEGF plays an important role in both physiological and pathological angiogenesis. Embryonic lethality in mice between day 11 and 12 following the loss of even a single allele of VEGF emphasizes the crucial role played by VEGF in embryonic vasculogenesis and angiogenesis (Carmeliet et al., 1996; Ferrara et al., 1996). VEGF is expressed at high levels by the placenta (Sharkey et al., 1993) and also by several normal adult tissues undergoing physiological angiogenesis, such as the proliferating endometrium (Cullinan-Bove and Koos, 1993) and the corpus luteum (Kamat et al., 1995). Furthermore, VEGF has been found to be responsible for angiogenesis occurring during wound healing (Nissen et al., 1998) and in ischemic conditions (Ferrara, 1995). The high expression of VEGF mRNA in many tumors (Plate and Mennel, 1995) and in the retina of patients with diabetic retinopathy (Duh and Aiello, 1999), overexpression of VEGF and its receptors in psoriasis (Detmar et al., 1994), and higher VEGF levels in the synovial fluid of rheumatoid arthritis patients (Fava et al., 1994) support the hypothesis that VEGF is a key mediator of angiogenesis associated with pathological conditions.

D. Angiopoietin-1 and VEGF in Angiogenesis

Angiopoietin-1 (Ang 1) is a recently identified ligand (Davis et al., 1996) selective for the endothelial cell-specific tyrosine kinase TIE-2. Gene knockout studies have shown that Ang 1 is required for the correct assembly of the vessel wall (Suri et al., 1996). Ang 1 and VEGF play distinct but essential roles in vascular development during embryogenesis: VEGF acts early during vessel formation, whereas Ang 1 has a later role in angiogenesis, perhaps involved in vessel maturation and stabilization by promoting endothelial cell attachment to the surrounding matrix and cells (Davis et al., 1996; Suri et al., 1996; Dvorak et al., 1999). When coadministered with VEGF in a corneal micropocket assay of neovascularization, Ang 1 augmented the formation of neovessels and resulted in an increase in total microvessel density as well as an increase in patent microvessels surrounded by SMCs (Asahara et al., 1998). Transgenic Ang 1 was shown to act as an antipermeability factor when provided chronically during vessel formation (Thurston et al., 1999). Comparison of transgenic mice overexpressing VEGF and Ang 1 in the skin revealed that VEGF-induced blood vessels were leaky, whereas those induced by Ang 1 were nonleaky. Vessels in Ang 1-overexpressing mice were resistant to leaks caused by inflammatory agents, even in the presence of excess VEGF. Furthermore, coexpression of VEGF and Ang 1 had an additive effect on angiogenesis and the vessels were leakage resistant (Thurston et al., 1999). This study raised the possibility that a combination of VEGF and Ang 1 could have additive effects in promoting angiogenesis in addition to the beneficial effect of forming nonleaky vessels. Abundant angiogenesis in double transgenic (K14-Ang 1/VEGF) mice suggested that the angiogenic action of VEGF could be uncoupled from its leakage-inducing effects by Ang 1 (Thurston et al., 1999). In another recent study, it has been shown that acute administration of Ang 1 protects the adult vasculature from leaking (Thurston et al., 2000). In this study, an adenoviral gene delivery approach was used to deliver Ang 1 to adult mice. The study found that preadministration of Ad-Ang 1 blocked plasma leakage, demonstrating that

short-term delivery of Ang 1 could make adult vessels resistant to leakage induced by either inflammatory agents or VEGF (Thurston et al., 2000). These findings seem to suggest that the process of neovascularization may involve a "cocktail" of growth factors that may act simultaneously or at different stages in the angiogenesis cascade to form functional blood vessels.

IV. PRECLINICAL STUDIES

To develop an effective therapy for angiogenesis, both protein- and gene-based approaches are being investigated. Recently, many studies have demonstrated augmented perfusion and development of collateral vessels in animal models of ischemia. Early evidence of the angiogenic potential of VEGF in vivo came from studies on the chorioallantoic membrane (Ferrara and Henzel, 1989), rat cornea (Levy et al., 1989), rabbit cornea, and the rabbit bone graft model (Connolly et al., 1989). To determine if the angiogenesis induced by VEGF in vivo is sufficient to constitute a therapeutic effect, Takeshita et al. (1994a) studied a rabbit model of chronic hind limb ischemia. In this model, ischemia is typically induced by ligation and excision of the femoral artery and its branches from the lower limb. An intraarterial administration of a single bolus dose of VEGF proximal to the site of ligation led to an increase in the capillary density and an improvement in the development of collateral vessels in the ischemic limb (Takeshita et al., 1994a). Preclinical studies using VEGF and other angiogenic factors in various animal models have subsequently been reported that document augmented collateral vessel development in the ischemic heart or limb (Table 2). Several parameters were evaluated as evidence for angiogenesis in these studies, including collateral vessel development, blood flow, muscle atrophy, capillary density, capillary per muscle fiber ratio, etc. These preclinical results established the potential therapeutic utility of VEGF in the treatment of conditions of vascular insufficiency. VEGF has also been demonstrated to prevent restenosis following angioplasty because of its ability to promote reendothelialization and thereby indirectly attenuate neointimal thickening due to SMC proliferation (Asahara et al., 1995).

bFGF also showed considerable therapeutic potential in stimulating collateralization. Daily intramuscular injections of bFGF increased collateral vessel development in a dose-dependent manner in the rabbit ischemic hind limb model (Baffour et al., 1992). Subsequent preclinical studies demonstrated the efficacy of bFGF in enhancing angiogenesis in the ischemic myocardium (Battler et al., 1993; Harada et al., 1994; Unger et al., 1994). While VEGF and bFGF independently enhanced collateral development in the rabbit ischemic hind limb model, their simultaneous administration showed a synergistic effect (Asahara et al., 1995).

The gene therapy approach has the advantage that it can provide local production of the protein at the target site for several days following gene transfer (Laitinen et al., 2000). Preclinical results in the ischemic rabbit hind limb model suggest that intraarterial as well as intramuscular injection of naked plasmid DNA encoding $VEGF_{165}$ ($phVEGF_{165}$) could improve blood flow and collateralization of the limb (Takeshita et al., 1996b; Tsurumi et al., 1996) (Table 3). The replication-deficient adenovirus vector, AdCMV.VEGF165, containing the cDNA for human $VEGF_{165}$ has been shown to induce angiogenesis following subcutaneous injection in a mouse model (Muhlhauser et al., 1995). Recent findings in rabbit iliac arteries also indicate that arterial VEGF gene transfer could effectively accelerate endothelialization of stents, thus decreasing occlusion because of in-stent thrombus formation and intimal thickening (Van Belle et al., 1997). The data from preclinical studies not only established the efficacy of both protein- and gene-based approaches in inducing angiogenesis but also documented their utility in improving limb and cardiac function in settings of ischemia.

V. CLINICAL TRIALS

Despite promising results in various animal models of limb and cardiac ischemia, the results of an early clinical trial were not encouraging. In an initial study in a nondiabetic elderly patient with leg ischemia, 2 mg of human plasmid $phVEGF_{165}$ was applied to the hydrogel polymer coating of an an-

TABLE 3
Preclinical Studies Using the Gene Approach for Therapeutic Angiogenesis

Angiogenic factor	Dose	Mode of administration	Animal model	Results and conclusions	Refs.
$phVEGF_{121}$ $phVEGF_{165}$ $phVEGF_{189}$	—	Percutaneous delivery after application of plasmid to the hydrogel polymer coating of an angioplasty balloon	Rabbit hind limb ischemia	Augmented development of collateral vessels suggesting that site-specific arterial gene transfer of $VEGF_{165}$ may achieve therapeutic angiogenesis	Takeshita et al., 1996a
$phVEGF_{165}$	500 µg	IM	Rabbit hind limb ischemia	Augmented collateral vessel development and tissue perfusion. IM gene transfection may be an alternative to intravascular gene therapy	Tsurumi et al., 1996
AdFGF-5	2×10^{11} viral particles	IC	Porcine myocardial ischemia	Improved myocardial function and regional blood flow associated with capillary angiogenesis	Giordano et al., 1996
phVEGF	400 µg	IM	Rat hind limb ischemia	IM gene transfer of VEGF causes endothelium-dependent relaxation of collateral microvessels	Takeshita et al., 1998
AdVEGF	4×10^{9} pfu	IM immediately after vessel ligation	Rat hind limb ischemia followed by acute vascular occlusion	Increased blood flow and vascularity in the ischemic limb in AdVEGF-treated rats. AdVEGF is capable of stimulating an angiogenic response that protects against acute vascular occlusion in the setting of preexisting ischemia	Mack et al., 1998

AdCMV.VEGF$_{165}$	6×10^9 pfu	Pericardial catheter	Canine model of progressive coronary occlusion	Adenoviral-mediated gene transfer is capable of inducing sustained VEGF$_{165}$ expression in the pericardium; however, locally targeted pericardial VEGF delivery failed to improve myocardial collateral perfusion in this model	Lazarous et al., 1999
Ad$_{GV}$VEGF121.10	10^{12} pfu	IMC vs. IC injection	Porcine myocardial ischemia	Vector delivery by IC administration is 33-fold less as compared with IMC delivery. Focal expression of VEGF following IMC administration	Lee et al., 2000
phVEGF$_{165}$	500 µg	IMC injection in the border zone of myocardial infarction	Rat model of myocardial ischemia	Injection of phVEGF-induced angiogenesis. Angioma formation at the injection sites did not appear to contribute to regional myocardial blood flow, which may be a limitation of gene therapy for this application.	Schwarz et al., 2000
AdVEGF	1×10^9 pfu	IM	Hind limb ischemia in diabetic mice	Diabetes impairs endogenous neovascularization of ischemic tissues by reduction in expression of VEGF; intramuscular adeno-VEGF gene transfer restores neovascularization.	Rivard et al., 1999b
phVEGF$_{165}$	500 µg	IMC	Porcine model of myocardial ischemia	Nonviral, plasmid-based IMC gene transfer results in sufficient VEGF production (peak at 4–5 days after gene transfer) to enhance myocardial perfusion in ischemic pig hearts.	Tio et al., 1999

Note: IM, intramuscular; IMC, intramyocardial; IC, intracoronary; pfu, plaque forming units; phVEGF, plasmid DNA encoding human VEGF; AdVEGF, adenoviral vector expressing cDNA for human VEGF.

gioplasty balloon and administered intraarterially to the ischemic leg (Isner et al., 1996). Transient peripheral edema was observed in the ischemic limb in 7 days. Though new collateral vessels were observed 1 month after gene therapy, limb gangrene could not be reversed, the neovessels regressed after 12 weeks, and the limb had to be amputated below the knee 5 months after gene therapy. In a more recent phase I clinical trial, angiogenesis was induced in 10 critically ischemic limbs in 9 patients by the direct intramuscular transfer of human plasmid phVEGF$_{165}$ (Baumgartner et al., 1998). Therapeutic efficacy was demonstrated by improved distal flow, healing of ischemic ulcers, limb salvage (in some cases), and reduction in rest pain. New collaterals were observed in 7 out of the 10 patients, yet 3 required below knee amputation (including one in whom no collaterals were observed). Another Phase I trial was initiated to determine the safety and efficacy of direct myocardial gene transfer of VEGF as sole therapy for patients with myocardial ischemia (Losordo et al., 1998). All 5 patients involved reported reduction in angina between 10 and 30 days after gene transfer. These trials using the plasmid encoding for VEGF$_{165}$, though not placebocontrolled, were considered partially successful in establishing the feasibility of therapeutic angiogenesis in the treatment of ischemic conditions (Table 4).

However, a placebo-controlled phase II clinical trial with recombinant human VEGF$_{165}$ failed to achieve its primary objective of improved exercise tolerance in patients suffering from coronary ischemia (Brower, 1999). The protein was administered as a single intracoronary infusion followed by three intravenous infusions, yet the treatment group did not show any improvement in treadmill time and pain relief over the placebo group at 60 days (Ferrara and Alitalo, 1999).

VI. ANALYSIS OF PRECLINICAL AND CLINICAL TRIALS

Though considerable therapeutic efficacy was observed even with very small amounts of VEGF in animal models of cardiac and limb ischemia, the protein did not show similar benefits in human patients. A possible explanation for this discrepancy has been proposed (Ferrara, 1999b). Whereas young and otherwise healthy animals are able to induce an effective endogenous angiogenic response to ischemia that can be potentiated by exogenous growth factors, the angiogenic response initiated by patients with extensive atherosclerotic disease may be much smaller. Therefore, very brief exposures to VEGF may not be sufficient to stimulate a therapeutically significant response, whereas a more persistent exposure might be effective (Ferrara and Alitalo, 1999). To address the issue of age as a factor in angiogenic response, Rivard et al., (1999b) have studied collateral vessel development following surgical induction of ischemia in young and old rabbits. In this study, angiogenesis was significantly impaired in older animals as a result of endothelial dysfunction and reduced VEGF expression, but both groups responded equally well to recombinant VEGF administration, suggesting that advanced age may not hinder collateralization in response to exogenous angiogenic agents. Another important discrepancy between the preclinical studies and clinical trials has been the dose and the duration for which the growth factor was administered. Many animal studies were carried out with higher doses and continuous administration of growth factors over several weeks. In humans, however, the risk of nonspecific toxicity limits the use of higher and repeated doses of such potent angiogenic agents.

VII. ISSUE OF DRUG DELIVERY IN ANGIOGENESIS THERAPY

Clinical results highlight certain key issues associated with angiogenesis therapy. Using recombinant VEGF protein, systemic administration may not provide a therapeutic concentration of the growth factor at the disease site. Furthermore, the growth factor may be rapidly degrading in the circulation (the biological half-life of VEGF is only a few minutes) and may therefore not be available at the disease site for a duration long enough to induce and sustain the growth of new collaterals. Spontaneous regression of the collateral neovasculature could be a problem without repeated treatments (Thompson et al., 1999). The protein demonstrated its angiogenic effect in various animal model studies when administered as

an intravenous continuous infusion at the site of ischemia or given as repeated intramuscular injections over several weeks (Banai et al., 1994; Hopkins et al., 1998). Banai et al., (1994) have demonstrated that an intracoronary administration of VEGF daily for 28 days enhanced collateral function in a canine model of myocardial ischemia. However, reducing the duration of administration to 7 days failed to show any effect, suggesting the importance of the sustained effect of growth factor for therapeutic results. Many studies have used an Alzet® pump (Alza Corp., Palo Alto, CA) to achieve a sustained delivery of the growth factor. Harada et al. have demonstrated improvements in blood flow and hemodynamic parameters with continuous administration of the growth factor using an Alzet® pump in the ischemic pig myocardium (Harada et al., 1996). In another study, an Alzet® pump was used to deliver VEGF intraarterially at a rate of 0.22 μg/kg/day for 28 days (total dose per animal 25 μg) to induce neovascularization in a rabbit hindlimb ischemia model (Hopkins et al., 1998). In this study, enhanced revascularization was obtained at a dose approximately 20- to 120-fold less than previous systemic doses. These studies thus demonstrate that a sustained presence of VEGF (~28 days) is required for a therapeutic angiogenic effect in different animal models.

Another important issue is the nonspecific effect of proangiogenic agents. Hypotension is a potential problem arising from systemic administration of high doses of VEGF. Hariawala et al. (1996) reported that intracoronary administration of a high bolus dose of VEGF (2 mg) in a porcine model of myocardial ischemia induced severe hypotension leading to mortality in 4 out of 8 pigs. The nonspecific effect of growth factors has also been reported in other studies. Asahara et al. (1996) found an increase in reendothelialization of balloon-injured vessels not only in the limb transfected with $phVEGF_{165}$ cDNA but also in the contralateral limb. This non-specific angiogenic effect could be a cause for concern in certain conditions. Most tumors in humans persist in situ in a state of dormancy until a subgroup of cells in the tumor "switches" to an angiogenic phenotype (Folkman, 1995b). The switch involves a change in the balance between positive and negative regulators of angiogenesis in the microvasculature. It is possible that systemic delivery of VEGF or its rampant expression can provoke dormant tumors in some patients to progress by turning on the "angiogenic switch" for these tumors. The low efficiency of gene transfer in most cell types by plasmid delivery, the inability to turn off VEGF production in case of complications, and the immune system response arising from the use of viral vectors are a few of the other limitations of the gene therapy approach (Majesky, 1996). A gene therapy trial using plasmid DNA for myocardial angiogenesis is currently on hold because of nonspecific toxicity concerns (Marshall, 2000). The recent death of a patient in a gene therapy trial using an adenoviral vector renewed concerns about the safety of viral vectors (Marshall, 2000). In addition, VEGF may aggravate retinopathy in diabetics, who comprise 2 to 5% of patients suffering from critical leg ischemia (Dormandy et al., 1999). Thus, systemic toxicity in the form of exacerbation of pathological angiogenesis (Ferrara, 1995), hypotension (Hariawala et al., 1996), and edema (Isner et al., 1996), arising from repeated administration of high doses, could limit the clinical usefulness of VEGF.

VIII. LOCALIZED SUSTAINED ANGIOGENIC THERAPY

Despite the potent angiogenic effect of VEGF and other growth factors, an efficient delivery mechanism that could target the protein or the expression vector to the diseased tissue and maintain a local therapeutic concentration for a prolonged period of time is essential. The intraluminal localized infusion of only simple solutions does not provide therapeutic arterial drug levels for an adequate period of time because of rapid washout (<24 hrs, with over 90% drug leaching out within 30 min) (Meyer et al., 1994). For example, in studies by Laham et al. (1999a), an intracoronary infusion of bFGF resulted in 0.89% cardiac uptake of growth factor at 1 hour, which then dropped to 0.05% in 24 hours, suggesting an inefficient uptake of protein by the target tissue and a rapid washout effect. To overcome the above limitations, various sustained delivery systems have been investigated (Kornowski et al.,

TABLE 4
Clinical Trials

Angiogenic factor	Dose	Mode of administration	Patients and study design	Results and conclusions	Refs.
$phVEGF_{165}$	2 mg	Applied on hydrogel polymer coating of an angioplasty balloon	Single elderly nondiabetic patient	Increased blood flow and collaterals, transient edema in the treated limb. First clinical report of therapeutic benefit of VEGF therapy	Isner et al., 1996
$phVEGF_{165}$	4 mg	Direct IM injection	Phase I: chronic limb ischemia and ischemic leg ulcers	Improved distal flow, healing of ischemic ulcers, limb salvage in 3 patients, occurrence of transient lower-limb edema. Established feasibility of using phVEGF for therapy	Baumgartner et al., 1998
$phVEGF_{165}$	125 µg	Direct IMC injection	Phase I: coronary artery disease untreated by conventional therapy	Reduction in angina in all patients. Established safety of direct myocardial transfer of naked plasmid DNA	Losordo et al., 1998
Human FGF-I	0.01 mg/kg	Intracardiac close to the vessels during surgery	Patients undergoing bypass surgery for multivessel coronary heart disease	Angiographic evidence of new capillary formation at the site of injection	Schumacher et al., 1998
rh-bFGF	10 µg 100 µg	Sustained-release heparin-alginate microcapsules implanted in ischemic myocardium during CABG	Phase I: patients undergoing CABG	All patients in the 100 µg group were angina-free and there were no major complications. The trial established safety and feasibility of this mode of therapy for coronary artery disease	Laham et al., 1999b

$phVEGF_{165}$	125 µg 250 µg	Direct IMC injection	Phase I: patients with "inoperable" coronary artery disease	Reduction in angina in all patients. One death at 4 months	Symes et al., 1999
$Ad_{GV}VEGF121.10$	4×10^9 pu	Direct myocardial injection	Phase I: Patients with reversible left ventricular ischemia	Three deaths—two unrelated to therapy, one of unknown cause. Vector administration appeared well tolerated at 6-month follow-up	Rosengart et al., 1999a, 1999b
$rhVEGF_{165}$	17 or 50 ng/kg/min each	Two 10 min. IC infusions + three 4-hour IV infusions	Phase II: placebo controlled: advanced coronary artery disease	No improvement in exercise tolerance of the test group over the placebo group	Genentech, Inc. (Brower, 1999)
$pVEGF_{165}$/ liposomes	1 mg	Intracoronary artery via perfusion-infusion catheter after PTCA	Placebo-controlled study: patients with angina undergoing PTCA	First report of transgene expression in human peripheral arteries following intracoronary gene transfer. No clinical effects detected in angiography 6 months after gene transfer	Laitinen et al., 2000

Note: $phVEGF_{165}$, naked plasmid DNA encoding human $VEGF_{165}$; rh, recombinant human protein; PTCA, percutaneous transluminal coronary angioplasty; IC, intracoronary; CABG, coronary artery bypass surgery; IMC, intramyocardial.

2000). A catheter tip was coated with a hydrogel and soaked in an expression vector prior to insertion into the artery (Isner et al., 1996). This approach is also ineffective because most of the protein on the catheter could get washed off prior to reaching the site of application, since hydrogels are water soluble. Furthermore, the transfer of the protein from the catheter tip to the arterial wall is less efficient (less than 0.4%) (Fram et al., 1994). In addition, the retention time of the drug transferred to the wall could be less than one hour. Calcium alginate beads were examined as a means of delivering bioactive VEGF at a controlled rate over extended times (Peters et al., 1998). The alginate beads demonstrated the ability to release encapsulated VEGF at a constant rate for up to 14 days in vitro. The released VEGF, when assayed for its mitogenic activity on endothelial cells in culture, was found to be three to five times more potent than the same mass of VEGF added directly to the culture medium. Calcium alginate microcapsules containing FGF-2, designed to deliver the growth factor over several weeks when implanted in the myocardium, demonstrated improved myocardial perfusion and contractility in the ischemic zone (Harada et al., 1994) and normalization of endothelium-dependent relaxation of the collateral-dependent microcirculation (Sellke et al., 1996). Hayashi et al. (1998) used Gelfoam® (Upjohn and Pharmacia, Kalamazoo, MI) as a sustained action delivery system to deliver VEGF locally to the brain following transient ischemia. The study demonstrated significant reduction in brain edema in animals that were treated with VEGF. Ozaki et al. (1997) used ethylene-vinyl acetate (EVA) copolymer pellets for sustained intravitreal delivery of the growth factor to demonstrate neovascularization in the retina. Recently, microparticles containing VEGF have been formulated using a biodegradable polymer, poly(DL-lactide-co-glycolide) (PLGA). These microparticles demonstrated sustained release of biologically active VEGF (King and Patrick, 2000). In another study, a similar formulation of PLGA microspheres loaded with VEGF has been shown to produce local neovascularization in a non-ischemic aged mouse model (Daugherty et al., 1999). The above studies thus demonstrate the feasibility of delivering VEGF in a controlled release drug delivery system.

IX. CONCLUSIONS AND FUTURE

Therapeutic angiogenesis using either recombinant genes or growth factors opens a new approach for the treatment of ischemia. As the biology of proangiogenic agents is revealed further, it is important that appropriate drug delivery systems are also developed, which could provide a therapeutic dose of growth factor in the target tissue for a period of time necessary for therapeutic angiogenic effect. Furthermore, site-specific sustained delivery systems could bridge the gap that usually exists between animal studies and humans in terms of the doses used. Such systems could achieve the therapeutic effect of growth factors at relatively lower doses without causing systemic toxicity. Similarly, a safe and efficient expression vector is needed for a gene therapy approach to angiogenesis. Unless due consideration is given to the fact that the biological instability of growth factors is a major problem, many such potent therapeutic agents are less likely to succeed in clinical trials. Developing an appropriate drug delivery system could be one way of solving the problem.

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Gene Therapy for Myocardial Angiogenesis: Has It Come of Age?

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Current Atherosclerosis Reports 2000, 2:373–379
Current Science Inc. ISSN 1523–3804

Vasculogenesis and angiogenesis are the processes responsible for the development of the circulatory system during embryonic and adult life. Vasculogenesis occurs during embryogenesis while angiogenesis refers to blood vessel formation from any preexisting vasculature. Postnatal angiogenesis resumes during reproduction, wound healing, and ischemia. Excess blood vessel formation may contribute to initiating and maintaining many diseases such as chronic inflammatory disorders, tumor growth, restenosis, and atherosclerosis. In contrast, insufficient blood vessel formation is responsible for tissue ischemia, as in coronary artery disease. An increasing number of patients with advanced coronary artery disease remain symptomatic despite maximal interventional, surgical or medical treatment. Ideally, they would benefit most from additional arterial blood supply to ischemic areas of myocardium. Therapeutic angiogenesis, the ability to induce the growth of new blood vessels, is one of the most intriguing new frontiers in interventional cardiology for this growing patient group. Several approaches are currently undergoing intensive experimental investigations or have already entered early clinical trials involving either local angiogenic peptide administration or the transfection of angiogenic genes. Gene therapy for therapeutic myocardial angiogenesis is the most promising synthesis of two emerging technologies. In the following article, we will review the fundamental pathophysiological concepts of gene-based angiogenic therapy, the technical approaches and delivery systems, and the results of the first clinical trials. We will also discuss the controversies and unresolved issues of this new revascularization therapy.

Introduction

Background

Chronic myocardial ischemia is a potent natural stimulus for the expression of angiogenic factors and growth factor receptors. These factors initiate the cascade of events leading to collateral vessel formation to protect ischemic tissue [1,2,3,4]. Even when maximal collateralization occurs, blood flow is usually not restored to normal levels. Moreover, collateral formation occurs to varying degrees in individual patients causing insufficient collateral formation in 50% of patients [2,5].

Gene-based therapy for myocardial angiogenesis opens new therapeutic options for patients with insufficient collateral formation by correcting genetic defects or by expressing gene products that are therapeutically useful [6]. It involves the transfer of nucleic acids to somatic cells of an individual with a resulting therapeutic effect. This approach is currently under investigation in experimental and early clinical trials as a treatment option for patients with severe peripheral and coronary atherosclerotic disease. Direct administration of the angiogenic factor requires repeated or prolonged administration of the protein. Gene transfer of the angiogenic factor provides a viable alternative to this expensive and invasive approach by providing a more sustained and localized delivery of the angiogene. Gene transfer targeted specifically to ischemic myocardium may give a long-lasting therapeutic effect and reduce side effects. The availability of gene therapy vectors, various angiogenic growth factors, and the demonstration of their angiogenic potential in animal models of chronic myocardial ischemia, has led to intensive investigation of their potential in clinical trials.

Basic concepts and mechanisms of angiogenesis

Angiogenesis, the ability to generate new blood vessels in response to a variety of conditions and triggers, is maintained throughout life [7,8]. This process involves matrix dissolution, cell migration, cell adherence and proliferation, tube formation, and capillary anastomosis. The endothelial cell represents the central element in the process of angiogenesis governed by stimulatory and inhibitory growth factors [3,7,9••]. Endothelial cell activation by secreted growth

Table 1. Vectors for gene transfer

Vector	Advantages	Disadvantages
Nonviral		
Plasmid DNA	Simple and widely available technology Very little toxicity, high level of safety Large-size DNA transfection possible	Low tranfection rates
DNA-liposomes	Simple technique Broad transfection cell range Little immunogenicity compared to viral vectors Larger DNA transfer possible compared to viral	Low efficiency and inconsistent transfection rates Little specificity Potentially cytotoxic to some cell lines
Protein-DNA complex	Large DNA transfer Cell-specific	Inefficient gene transfer Immunogenic
Viral		
Retrovirus	Stable transfection Potentially long-term gene expression after integration into host DNA	Transfection only in dividing cells Size limitations for gene inserts
Adenovirus	Most widely used approach High transfection efficacy in dividing and nondividing cells Production in higher titers than retrovirus	Vector grown in low titer Nonspecific transfection Inflammation and immunogenic response
Adeno-associated virus	Less immunogenic than adenovirus Longer transgene expression Nonpathogenic	Limited DNA size Low titers Requires replicating adenovirus to grow Technology not widespread

factors initiates the angiogenic process. Ischemia and inflammation upregulate growth factor receptor expression [9••]. Local vasodilatation increases vascular permeability and accumulation of perivascular fibrin around the parent blood vessel ensues. These events promote the influx of inflammatory cells, which secrete additional growth factors and cytokines, thus amplifying the process [3]. The vascular basement membrane becomes disrupted, thus facilitating subsequent migration of endothelial cells. There is concomitant proteolytic dissolution of the extracellular matrix surrounding the parent vessel providing space for endothelial cell migration and for the growth of new vessels. Endothelial cells from the parent vessel proliferate and migrate towards the ischemic stimulus. They organize to form elongating solid cylindrical extensions from the parent vessel. Simultaneous migration and proliferation of vascular smooth muscle cells and fibroblasts occasionally occurs to form arterioles. An intravascular lumen forms and the newly formed capillaries anastomose. The surrounding extracellular matrix regenerates. The process is completed when the endothelium achieves functional maturation.

The concept that stimulation of coronary collateralization by an angiogenic factor reduces damage to ischemic myocardium was first demonstrated in a canine myocardial infarct model [10••]. Two weeks after two intracoronary injections of basic fibroblast growth factor (bFGF), the number of arterioles and capillaries in the infarcted area significantly increased. Treatment with bFGF in this model also improved cardiac systolic function and reduced infarct size. The functional significance of growth-factor induced angiogenesis in chronic ischemia has been demonstrated in a porcine model. Six weeks after delivery of a polymer, which slowly released bFGF to the adventitial side of the occluded coronary artery, there was significant neovascularization, enhanced blood flow, and improved global and regional left ventricular function in the treatment group [11]. Many other animal experiments were able to confirm these results demonstrating that administration of an angiogenic growth factor is itself sufficient to augment blood delivery [12–14].

Growth Factors for Therapeutic Angiogenesis

A large variety of naturally occurring cytokines and growth factors can induce or promote angiogenesis by stimulating endothelial cell growth and migration (Table 1). Vascular endothelial growth factor (VEGF) is an endothelial specific mitogen which shows receptor upregulation in ischemic tissue. Angiopoitein-1 and angiopoitein-2 have been identified as factors that may modify the response of VEGF gene therapy by affecting maturation and stability of the newly formed vessels. Angiopoitein-1 mediates the recruitment of smooth muscle cells to the new vessel wall, while angiopoitein-2 appears to prevent or downregulate smooth muscle apposition to the walls of microvessels [15]. VEGF has been the most widely used agent for therapeutic angiogenesis [13]. Many animal models and initial unrandomized clinical studies have demonstrated beneficial effects of VEGF in ischemic myocardium.

The fibroblast growth factor (FGF) family induces angiogenesis in vivo by affecting various cell types including endothelial cells, and smooth muscle cells [14]. FGFs, like VEGF, bind heparin-like components of the extracellular

matrix (heparan sulfates) on the surface of endothelial and smooth muscle cells thereby facilitating attachment of these growth factors to their receptors. In various ischemic animal models, FGF induced myocardial angiogenesis and increase local perfusion [10,14,16]. Preliminary clinical data have shown promising effects with increased microvessel density and subjective improvement of symptoms [17•]. Receptor expression of these growth factors varies according to the vascular bed and according to oxygen tension. There are probably other alternative mechanisms inducing angiogenesis since blood vessel development also occurs in areas with normal oxygen tension.

Other cytokines such as monocyte chemotactic protein-1, hepatocyte growth factor (HGF), and platelet derived growth factor appear to have indirect angiogenic activity probably by stimulating VEGF secretion in vivo [18]. The angiogenic cascade is still incompletely understood and it is very likely that more angiogenic factors for therapeutic angiogenesis will be identified in future. Gene therapy appears to be the best way of inducing myocardial angiogenesis, since sustained but transient growth factor expression is required for the formation of new blood vessels.

Vectors

Ideally, a vector for gene transfer should be easily produced with a broad range of transgene size. It should be tissue-specific with minimal toxic side effects, nonimmunogenic, and provide sustained expression. A multitude of vector systems are currently under evaluation for the introduction of genetic material into human tissue especially myocardium (Table 2). Nonviral transfer and viral gene transfer are the two major categories of transfer considered for gene-based therapeutic angiogenesis.

When naked plasmid DNA is applied to cell membranes, only a very small amount will pass into the cell, leading to a low gene-transfer efficiency. This approach is technically simple and has low toxicity, but efficiency is typically less than 1%. The expression duration is only transient. Therefore, carrier molecules such as liposomes, proteins or polymers are generally used to enhance efficacy [19,20]. Cationic liposomes interact with negatively charged plasmid DNA to form complexes able to fuse with cell membranes, releasing plasmid DNA into the cytoplasm [20,21]. Transfection rates range between 0.5% and 2% in vivo. When used to mediate expression of a potent secreted molecule such as VEGF plasmid-based gene transfer may have biological effects. In a phase I clinical trial, patients with severe peripheral artery disease improved clinically after local plasmid-based gene transfer of $VEGF_{165}$.

Viral vectors such as retroviruses, adeno-associated viruses (AAVs), and adenoviruses are considerably more efficient for angiogenic gene transfer. Retroviruses enter the cell via receptors and integrate into the host genome [22]. Retroviruses are only effective for dividing host cells leading to stable but low myocardial expression compared to adenoviruses. Therefore, most gene transfer to date uses adenovirus-based vectors.

Adenoviruses enter the cytoplasm via specific receptors. They remain extrachromosomal and cause a transient but effective expression in proliferating and nonproliferating cells. They can be produced in very high titre and have successfully been used for gene transfer to the myocardium [13]. First-generation adenoviruses produced significant immunologic and inflammatory reactions. This complication should be lessened with second-generation viruses [23]. Several approaches have been suggested to reduce the immune response to viral vectors including the administration of immunosuppressant drugs, cotransfection of vectors expressing immunomodulatory genes, or using andenoviral vectors with complete deletion of all transcriptional coding regions [24].

Tissue-specific gene expression may be achieved by using a myocyte-specific promoter to drive the transgene and to exclusively activate transcription in myocardium [25]. Viral vectors can be designed tissue-specific by modifying surface proteins that interact with cell surface receptors. Plasmid DNA can be applied tissue specifically by receptor-mediated gene delivery. A ligand of a myocardium-specific receptor antibody can be conjugated to a coupling agent to deliver DNA [24].

An intriguing concept of regulating transgene expression can be achieved by endogenous stimuli such as hypoxia especially for therapeutic angiogenesis. Placing a gene under the control of a hypoxia inducible promoter may allow growth factor production only in an hypoxic or ischemic environment [26]. The modulated interaction between the transcriptional complex "hypoxia-inducible factor-1" with its DNA recognition site may be useful in directing gene expression specifically to ischemic myocardium thereby limiting unwanted side effects.

The most effective formulations for sustained and controlled release of growth factors are those that mimic natural means of growth factor metabolism. Understanding the biochemistry of the natural means of optimized drug release will help in creating the best delivery strategies. Further development of vectors is clearly needed to optimize clinical studies and specific needs in pathologic tissue.

Technical Delivery Strategies

The effectiveness of gene therapy depends on gene expression of the transfected gene, entry of the genetic material into the cell, and the effects of gene delivery into the target tissue. Local delivery of a therapeutic agent to a specific region with minimal systemic toxic effects is the goal of optimal delivery systems. Genes encoding growth factors can be introduced into the myocardium via the following approaches: 1) intravascular injection of the transgene either by intracoronary, intravenous or intracoronary sinus application; 2) direct gene transfer to the ischemic region of the myocardium either during open-chest surgery or

Table 2. Angiogenic factors

Angiogen	Characteristics	Effects on endothelial cells	Effects on smooth muscle cells
FGF family (FGF-1–FGF-9, FGF-1 and -2 most important for therapeutic angiogenesis in humans)	Largest angiogen family, high homology also across species, concentrated in the extracellular matrix, bind heparin, heparan proteoglycan, in vivo angiogenesis, demonstrated for aFGF and bFGF only	Migration and proliferation	Migration and proliferation
FGF-1 (aFGF)	Mitogenic for a wide variety of cells, hypoxia induced upregulation	Migration and proliferation, stimulates endothelial cell chemotaxis and tubular formation	Migration and proliferation
FGF-2 (bFGF)	Upregulates VEGF, hypoxia induced upregulation	Migration and proliferation	Migration and proliferation
VEGF family	Most specific angiogen, four isoforms (VEGF121, 165, 189, 206), chemotactic for monocytes, enhances vascular permeability, in vivo angiogenesis demonstrated for VEGF121 and VEGF165	Migration and proliferation, mitogenic exclusively for endothelial cells, receptor expression almost exclusively restricted to vascular endothelial cells	Not involved
VEGF121	Secreted protein, not cell associated, binds heparin poorly	Migration and proliferation	Not involved
VEGF165	Most abundant form in human myocardium, binds heparin	Migration and proliferation	Not involved
TNF-alpha	Indirect angiogenic activity by enhancing endothelial cell dependent bFGF production, in vivo angiogenicity bimodal and dose-dependent, chemotactic for monocytes	Indirectly involved	Not directly involved
TGF-alpha	Phenotypic endothelial cell modulation, enhances endothelial cell DNA synthesis	Proliferation	Not involved
TGF-beta	Upregulates PDGF, VEGF and bFGF, extracellular matrix production necessary during angiogenesis, chemotactic for monocytes	Proliferation, bimodal effect	Proliferation, bimodal effects

using a catheter-based endocardial injection device; and 3) intrapericardial gene application.

Intravascular infusion of a gene encoding a growth factor has been explored at every level of the myocardial vasculature including intracoronary, intravenous or intracoronary sinus administration. Giordano [14] pioneered this approach by intracoronary injection of a recombinant adenovirus expressing human FGF-5 in an animal model of stress-induced ischemia. Treated myocardium showed messenger RNA and protein expression and evidence of angiogenesis. Two and 12 weeks after gene transfer, regional ventricular function and blood flow had improved in the ischemic region.

Based on promising results with retrograde drug delivery through the coronary venous system, this route has been explored for gene-delivery [27]. Intracoronary and intracoronary venous delivery of a replication-deficient adenoviral vector expressing two reporter genes was compared during brief ischemia and without ischemia. At seven days, intravenous gene-delivery resulted in significantly higher expression than intracoronary application. Combined retrograde and antegrade gene-infusion showed similar expression levels to retrograde infusion alone.

A number of approaches and devices have been proposed for site-directed intramyocardial application including epicardial injection during bypass surgery or a catheter-based endocardial method [17•,28,29]. The theoretical advantage of this route may be that less genetic material will be delivered to other organs, although current injection devices show a high percentage of washout [28].

The catheter-based approach is less invasive and therefore safer than the sole surgical access.

Catheter-based intramyocardial and intravascular delivery strategies are associated with rapid washout of the agent, which may have led to negative outcome of clinical trials. As an alternative to local intracoronary or direct myocardial injection methods, the pericardial space has been explored as a suitable delivery area to the heart [30,31]. Intrapericardial delivery has been performed in experimental animal models using a surgical and percutaneous transarterial or transventricular approaches. Laham *et al.* [31] administered a single dose of bFGF (30 μg, 2mg or saline) into the pericardial space of pigs three weeks after induction of myocardial ischemia by an ameroid constrictor. Both bFGF groups showed improved myocardial perfusion, regional function, and endothelium-dependent vasodilatation compared to the placebo group receiving saline. Recently, March *et al.* [30] demonstrated highly efficient adenovirus vector delivery, gene transfer and expression in the pericardium using a catheter-based transmyocardial approach in dogs. The benefits of delivering therapeutic agents to the pericardial sac include enhanced consistency of local growth factor levels, markedly reduced acute systemic delivery and prolonged local exposure of myocardial tissue to the therapeutic material as a result of the reservoir function of the pericardial sac [32]. It is unclear whether this approach will be feasible in patient with end-stage coronary artery disease with multiple previous coronary artery bypass surgery.

The ideal delivery strategy is unknown at this point. Unresolved issues regarding delivery strategies concern single bolus versus repeated administration, local versus systemic delivery, and whether the combination with other interventional procedures will improve clinical outcome.

Clinical Trials

Multiple animal studies have proven the concept of enhancing angiogenesis in ischemic myocardium. A decade after the initial experiments, the first clinical data of myocardial gene transfer are available. Intramuscular VEGF and FGF gene transfer into arteries or muscle of an ischemic limb and into ischemic myocardium has shown beneficial effects [17•,33,34]. In an uncontrolled phase I clinical trial, direct myocardial injection of naked plasmid DNA encoding VEGF via a minimally invasive thoracotomy resulted in significant reduction in angina, and improved perfusion assessed by dobutamine SPECT-sestamibi imaging. Coronary angiography showed improved Rentrop score in all patients [33].

Gene-based therapy has been used as a hybrid procedure during incomplete bypass surgery in patients and in combination with transmyocardial revascularization (TMR) in animals. Intramyocardial, and periadventitial FGF injection during bypass surgery in patients with non-revascularizable territories improved collateral formation [17]. The combination of TMR and gene-based angiogenic therapy has only been performed in animals. In an canine infarct model, bFGF administration significantly enhanced the angiogenic effect of transmyocardial laser revascularization [16].

Despite these encouraging preliminary results, the first randomized, blinded clinical trial of intracoronary and intravenous injection of recombinant VEGF (VIVA-trial) did not improve outcome in patients with severe angina [35]. At 60 days, however, there was evidence of a dose-dependent effect on myocardial perfusion in a subset of patients. These results need to be reevaluated and confirmed in larger randomized trials and appropriate growth factor dosages need to be defined. Future trials should use standardized imaging protocols, because the choice of imaging protocol has greater impact on the results than does the choice of imaging modality [36].

A major problem in all clinical trials of angiogenic gene therapy is measuring treatment efficacy. Symptomatic improvement in quality-of-life or anginal symptoms are subjective endpoints and cannot be used as sole efficacy endpoints [28]. It has been difficult to demonstrate and quantify improved perfusion of angiogenic therapy using exercise tests, nuclear scans, stress echocardiography, and angiographic collateral assessment [37]. PET scan has successfully been used after VEGF administration in a porcine model [38]. MRI could be a promising imaging tool in the future, once it has been validated [39]. Recently, SPECT images evaluated with a modified semiquantitative 20-segment scoring method have shown a dose-dependent effect of intracoronary recombinant human VEGF in 14 patients [37]. These early positive results require additional validation in larger, prospectively, possibly blinded designed clinical trials. The results of ongoing trials will help shaping the nature and the timing of future studies. Negative results in initial phase I and II clinical trial should not discourage from trial completion and from further clinical research in the field. More background knowledge on myocardial angiogenesis will help improve clinical study designs.

Ethics and Safety of Gene Therapy

Side effects of gene-based angiogenic therapy are procedure related, growth factor related, and vector related events. Catheter-based delivery strategies to achieve myocardial angiogenesis are probably the safest approach and carry a risk comparable to other percutaneous interventions [28].

Animal experiments and early clinical trials have demonstrated the safety and feasibility of gene-based release of angiogenic factors. One potential problem associated with the intravascular administration of VEGF is potent nitrous oxide (NO) release causing peripheral vasodilatation and systemic hypotension. Intramyocardial delivery can minimize this effect. bFGF appears to act independently of NO secretion, but may stimulate intra-

coronary plaque growth [40]. Intramuscular VEGF gene therapy for peripheral atherosclerosis occasionally induces leg edema and hemangioma formation [13]. Other potential risks of therapeutic angiogenesis are development of non-functional leaky vessels, stimulation of angiogenesis in malignant tumors and retinopathy, intraplaque hemorrhage, and plaque rupture [18].

All gene therapy trials for myocardial angiogenesis are based on local delivery and somatic cell transfection [13]. The risk of inadvertent germline transmission is minimal, because only a transient expression of the transgene is required to achieve a local therapeutic effect. However, it should be recognized that serious adverse and even lethal effects may accompany adenovirus-mediated gene therapy [41]. Future trials must optimize inclusion and exclusion criteria for this new therapy. If safety and efficacy of gene therapy for myocardial ischemia can be established in the future, there should be no fundamental ethical issues for this new therapy especially in the elderly non-reproductive population.

Conclusions

Angiogenic gene therapy is an new treatment option for patients with severe occlusive atherosclerotic coronary and peripheral artery disease refractory to medical therapy and not amenable to mechanical revascularization. Animal studies have proven the feasibility of stimulating collateral formation and their function by gene-based delivery of angiogenic factors. Early clinical trails have demonstrated safety and feasibility if the gene material is administered intravascularly or intramyocardially. Future clinical trials will give more insight into appropriate dosage and must address efficacy. More agreement on end points to assess angiogenic efficacy is needed. It seems reasonable to choose physiologic endpoints (global and regional left ventricular function) as well as clinical parameters (angina frequency, symptom class, exercise time, and total and cardiovascular mortality).

Major challenges in future include improving viral vectors, the immunologic tolerance, safety, and transgene expression. Modern technologies like DNA-chips and the completion of the human genomic project will help identifying more angiogenic factors, which could potentially be used in combination to mimic a more physiologic response. Sequential administration of angiogenic genes may be more effective that a single application of one growth factor. More information is needed on the molecular details of angiogenesis finally leading to persistent functional collateral vessels. Very little is known about the interindividual heterogenity of gene expression in different patient populations, which may affect the success of gene-based therapy significantly. The promise of gene therapy for myocardial angiogenesis remains high. The development of gene therapy for therapeutic angiogenesis will proceed rapidly in the next years because of its great importance for coronary artery disease.

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Thank you

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Thromb Haemost 2001; 86: 172–7

Gene Therapy in Hemophilia: Clinical Trials Update

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Key words

Hemophilia, gene therapy, retrovirus, adeno-associated virus, adenovirus

Summary

Disorders caused by inborn genetic errors have been a primary target for treatment by gene transfer. Hemophilia A and B have been considered especially important targets because the genes for factor VIII and IX are well characterized, levels of factor VIII and IX do not require complex regulation, small increases in factor level would have significant clinical benefits, good clinical and laboratory tests of efficacy exist, and excellent animal models of hemophilia are available. Four clinical trials of gene transfer in hemophilia, two in hemophilia A and two in hemophilia B, are currently underway or have been completed and two other trials have been approved. The collective interim results from these trials indicate that the current approaches and doses are safe and that low levels of expression are detected. These studies support the continued development of gene transfer as a potential treatment option for hemophilia.

Introduction

Recent reviews have highlighted the remarkable advances in that have placed hemophilia at the forefront of candidate diseases for gene therapy (1-7). In the wake of these advances, new clinical trials of gene therapy in hemophilia were initiated in 1999. Four clinical trials are currently underway or have been completed and two additional trials have received final approval by regulatory agencies. The purpose of this review will be to examine the cumulative results obtained to date in these clinical trials and to present current advances in the field which promise to lead to future developments in the treatment of this disorder.

Clinical Trials in Hemophilia A and B

Chinese trial in hemophilia B. The first clinical trial in hemophilia was reported from Fudan University and Changhai Hospital in Shanghai, China (8). The protocol was the sixth protocol approved by the NIH and the clinical trial was initiated December 3, 1991. This was a phase I trial using *ex vivo* retrovirus-mediated gene transfer in autologous skin fibroblasts (9). Two viral vectors were used: XL-IX, a Moloney murine leukemia virus based vector in which factor IX expression was under control of the viral long terminal repeat (LTR), and N2CMV-IX, in which factor IX expression was under control of the CMV promoter. Factor IX production by fibroblasts infected with either vector averaged 3420 ng factor IX per 10^6 cells per day.

Two brothers with hemophilia B, ages 9 and 13, were studied. The subjects had moderately severe 'cross reacting material' (CRM) negative hemophilia B with baseline factor IX activity and antigen levels of 2%. Skin fibroblasts obtained by biopsy were maintained in culture and transduced with XL-IX or N2CMV-IX. The resulting cells (HBSF-IX) secreted human factor IX at high levels *in vitro*. Following demonstration of *in vitro* synthesis of factor IX, the HBSF-IX cells were mixed with sterile rat tail collagen and injected subcutaneously into multiple sites in the wall of the abdomen and the back. Three sets of injections were carried out at approximately monthly intervals. A total of 6.6-11 $\times$ 10^8 cells were injected. No treatment-related side effects were observed. An increase in factor IX was observed as soon as 15-20 days after the first set of injections. After completion of the three sets of injections, plasma factor IX protein and activity in both patients was increased over 2-fold and persisted for more than 420 days. The bleeding tendency was reported to be decreased. One subject received an additional set of injections of HBSF-IX cells at 16 months after factor IX levels had diminished and demonstrated another increase in factor IX levels in response to the injections.

Transkaryotic therapy, inc (TKT) trial in hemophilia A. The first US clinical trial to start, the TKT trial was initiated in November 1998. This was designed as a phase I, open label, dose escalation study which employed an *ex vivo* approach similar to that used in China (10). While the overall approach is similar to that used in the Chinese trial, the TKT trial used transfection with a non-viral, plasmid vector to insert factor VIII into the cellular genome, rather than a viral vector. The plasmid contained the cDNA for the B-domain deleted form of human factor VIII under control of the CMV promoter.

A total of six subjects were studied, ranging in age from 20-72 years. Four of the six subjects were HIV seropositive without AIDS and all were hepatitis B or C virus seropositive with stable liver function. Dermal fibroblasts obtained by excisional skin biopsy were electroporated with the plasmid containing the B-domain deleted human factor VIII cDNA. Fibroblasts expressing the factor VIII gene were clonally expanded, characterized for the stability of the integrated gene sequences and production of factor VIII, and administered by laparoscopic omental injection performed under general anesthesia. A total of 100-400 million cells were injected per subject. At the last analysis reported in December 2000, follow-up in the six subjects ranged from 12-18 months. There were no serious adverse events. No inhibitors to factor VIII were reported. Three of the six subjects demonstrated repeated levels of factor VIII above baseline, increasing to 1% to 2% of normal, with a maximum of 4% (10), although the relation of the factor VIII levels to exogenous treatment were not defined.

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The initial results from this trial indicate that *ex vivo*, non-viral gene transfer is safe. The implantation procedure was well tolerated with no serious adverse events. Low levels of factor VIII expression were observed out to 18 months, although the temporal relationship between the increased factor VIII levels and administration of exogenous factor VIII are not clear.

Chiron trial in hemophilia A. The Chiron study was approved by the FDA on March 12, 1999 and the first patient was enrolled on June 1, 1999. This was designed as an open-label, multi-institution, single-dose, dose-escalation, phase I trial in volunteers with severe hemophilia A utilizing a replication-deficient Moloney murine leukemia virus (MoMLV) derived vector to deliver B-domain deleted human factor VIII cDNA [hFVIII(V)]. The vector is complement resistant and employs a human packaging cell line system with reduced homology between the retroviral vector and the packaging components, thus allowing production of high titer virus production without the generation of replication-competent retrovirus (11). Production of the B-domain deleted factor VIII is driven by the retroviral long terminal repeats (LTRs). In tissue culture, production of factor VIII using this system (1 $\mu g/10^6$ cells/24 h) compared favorably with production of factor VIII using tissue-specific adenoviral vectors. Preclinical studies in rabbits, normal dogs, and hemophilia dogs demonstrated persistence of factor VIII antigen levels for as long as 65 weeks (12).

Based on the results from the preclinical studies, an open label, phase I, dose-escalation study of [hFVIII(V)] was undertaken in subjects over the age of 18 years with severe hemophilia A and no inhibitor. Subjects could be HIV seropositive or seronegative, but were required to have stable CD4 counts over 400/mm^3 and were excluded if taking reverse transcriptase inhibitors because of their effect on reverse transcription of the [hFVIII(V)]. Five doses, 2.2×10^7, 9.2×10^7, 2.2×10^8, 4.4×10^8, and 8.8×10^8 transduction units/kg, were administered in divided daily doses via peripheral vein injection. A total of thirteen subjects were enrolled, three at each of the first four doses and one at the highest dose. The mean age of the 13 subjects was 37.5 years, with a range of 18-55 years. All 13 subjects were hepatitis C antibody (anti-HCV) seropositive; 5 were HIV seropositive. In the last interim analysis in September 2000, the median time on study was 36 weeks (range 1-54 weeks). Two subjects dropped out of the study for personal reasons, one after 3 and one after 6 months. The infusion of [hFVIII(V)] was well tolerated, with no serious adverse events. Liver function tests and complete blood counts showed no significant changes from baseline and no acceleration of chronic HIV or HCV disease was apparent. All tests for replication competent retrovirus (RCR) were negative. No FVIII inhibitor activity was detected. While no subject had sustained levels >1%, six patients showed FVIII >1% on at least two occasions 5 or more days after infusion of exogenous FVIII. Most elevated levels were in the range of 1.0-1.8%, although isolated levels of 2.3, 3.0, 4.3, and 6.2 were reported for 3 subjects. Elevated levels of factor VIII were detected as early as 8 days following treatment. Peripheral blood mononuclear cells (PBMC) demonstrated the presence of vector gene sequences by PCR testing to six months in all 10 subjects tested and to one year in 3 of 4 subjects tested. All of the four subjects tested at one year were in the two lowest dose groups, indicating persistence of vector sequences even at the lowest dose. Pharmacokinetic examination following the infusion of exogenous FVIII 13 weeks after vector infusion showed a statistically increased half-life (T1/2) and area under the curve (AUC) compared to pre-study values. Bleeding frequency was decreased in six subjects compared to historical rates.

Retroviral vectors such as [hFVIII(V)] are random integrating vectors. Integration promotes long term expression of the transduced gene, but there are potentially undesirable effects of random integration. A theoretical concern is the possibility of insertional mutagenesis, where integration occurs in an existing gene, causing disruption of the gene. When this occurs, for example, in a tumor promoter gene, the result is absent expression of the tumor promoter in the cell and, perhaps, reduced neoplastic potential. When this occurs in a tumor suppressor gene, the result might be the opposite. Despite this potential concern, no consequences of possible integration have been observed in hundreds of volunteers receiving gene transfer mediated by retroviral vectors. No unusual events or complications have been observed in this study. Another concern with integrating vectors is the possibility of germline transmission. The ability to integrate into the human genome raises concerns that DNA might reach gonadal tissue and insert into the germ cell genome, causing possible transmission to subsequent generations. Semen samples were examined at regular intervals during the Chiron study to assess for germline integration. Vector sequences were detected by PCR. A single, transient, low-level positive signal was detected in a single semen sample from one subject at week 9; all preceding samples and four subsequent samples in the subject were negative. There was insufficient sample to repeat the abnormal result, so it remains unclear whether or not this was a false positive. Kazazian has estimated that the natural rate of endogenous insertional mutagenesis in man is about one in 10-100; that is, approximately one individual in every 10 to 100 will carry a mutational insertion, a number much larger than the calculated rate for gene therapy (13). There was no evidence for germline transmission with [hFVIII(V)] in rabbits (14).

In summary, interim results from this phase I trial using a Moloney murine leukemia retroviral vector to deliver B-domain deleted human FVIII cDNA demonstrate that [hFVIII(V)]: 1) was safe at the doses and route of administration used, 2) persisted in PBMC as long as one year, 3) was associated with measurable Factor VIII levels in some individuals, and 4) was associated with increased available plasma FVIII (increased T1/2 and AUC) after infusion of exogenous FVIII.

Avigen trial in hemophilia B. The Avigen trial was approved by the FDA on May 24, 1999 and the first patient enrolled June 11, 1999. This was designed as a prospective, multi-institution, single dose, dose escalation, phase I trial to examine the safety of an adeno-associated virus vector to deliver human factor IX in subjects with severe hemophilia B. Coagulin-B, the recombinant AAV produced by Avigen which is used in the study, is derived from serotype AAV-2 and was produced by a triple transfection method using three plasmids: one carries the recombinant AAV genome consisting of the viral ITRs, the cytomegalovirus promoter, an intron, the factor IX cDNA, and a polyadenylation signal; a second, contains the wild type AAV cap and rep genes; and a third, contains adenoviral helper functions. Vector production was in HEK-293 cells and the recombinant virus was purified by multiple rounds of cesium chloride density gradient centrifugation. The adeno-associated virus was devoid of adenovirus.

The clinical trial with AAV was supported by the most extensive preclinical studies of any of the ongoing trials and was the only study in which species specific gene transfer was examined (15-17). Stable expression of human factor IX in C57Bl mice was demonstrated in muscle for at least three months but systemic expression was blocked by the development of antibodies. Similar studies in immunodeficient Rag-1 mice showed systemic expression of high levels of human factor IX for at least six months and of lower levels for the lifetime of the

Table 1 Clinical trial summary

	subject	age	dose	inhibitor	peak factor[Φ]
Chinese Study – hemophilia B *Ex vivo* transduction	1	9	$11x10^8$ cells	no	5.92%
	2	13	$6.6x10^8$ cells	no	4.13%
TKT Study – hemophilia A *Ex vivo* transfection	1	N/A	$1-4x10^8$ cells	no	N/A
	2	N/A	$1-4x10^8$ cells	no	N/A
	3	N/A	$1-4x10^8$ cells	no	N/A
	4	N/A	$1-4x10^8$ cells	no	N/A
	5	N/A	$1-4x10^8$ cells	no	N/A
	6	N/A	$1-4x10^8$ cells	no	N/A
Chiron Study – hemophilia A Retrovirus	2-1	50	$2.8x10^7$ TU/kg	no	1.8%
	5-1	33	$2.8x10^7$ TU/kg	no	<0.1%
	5-2	30	$2.8x10^7$ TU/kg	no	3.0%
	3-1	51	$9.2x10^7$ TU/kg	no	<0.1%
	3-2	55	$9.2x10^7$ TU/kg	no	1.3%
	5-3	50	$9.2x10^7$ TU/kg	no	6.2%
	2-2	20	$2.2x10^8$ TU/kg	no	<0.1%
	3-3	43	$2.2x10^8$ TU/kg	no	<0.1%
	5-4	18	$2.2x10^8$ TU/kg	no	4.3%
	1-1	52	$4.4x10^8$ TU/kg	no	<0.1%
	5-5	48	$4.4x10^8$ TU/kg	no	1.4%
	5-6	18	$4.4x10^8$ TU/kg	no	<0.1%
	5-7	20	$8.8x10^8$ TU/kg	no	N/A
Avigen Study – hemophilia B AAV	1	38	$2x10^{11}$ vg/kg	no	3.7%
	2	23	$2x10^{11}$ vg/kg	no	0.8%
	3	67	$2x10^{11}$ vg/kg	no	<0.3%
	4	29	$5x10^{11}$ vg/kg	no	<0.1%
	5	44	$5x10^{11}$ vg/kg	no	<0.1%
	6	43	$5x10^{11}$ vg/kg	no	1%
	7	38	$2x10^{12}$ vg/kg	no	N/A
	8	30	$2x10^{12}$ vg/kg	no	N/A

[Φ]Peak factor defined as the highest level observed in those individuals who demonstrated levels greater than 1% on at least two occasions, five or more days after infusion of exogenous factor. Individuals who did not demonstrate levels greater than 1% on at least two occasions, five or more days after infusion of exogenous factor are shown to have basal levels.

AAV, adeno-associated virus; TU, transduction units; vg, vector genomes; N/A, not available.

mice. The mouse studies were followed by studies of AAV medicated gene transfer of canine factor IX in dogs with hemophilia B. Sustained expression of factor IX for as long as 17 months at levels up to 70 ng/ml (0.02 U/ml) was demonstrated when hemophilia B dogs were infected with AAV containing the cDNA for canine factor IX.

The initial report from this trial describing the first three subjects demonstrated the safety of the AAV vector approach in human subjects (18). The three subjects, one with CRM (cross-reacting material) positive severe hemophilia B and two with CRM negative severe hemophilia B, received 2×10^{11} vg/kg. Vector was administered under general anesthesia through ultrasound-guided injections in the vastus lateralis muscle of the leg. The number of injections ranged from 10-72, depending on the dose administered. Muscle biopsies performed 8 to 12 weeks after vector administration showed immunohistochemical evidence for factor IX in the extracellular space in a pattern similar to that observed in preclinical studies. There was no muscle injury or inflammation. Although all three subjects had detectable pre-treatment titers of neutralizing antibodies against AAV that ranged from 1:10-1:1,000, gene transfer and expression was demonstrated in each subject by Southern blot on DNA extracted from muscle and by RT-PCR. Southern blot analysis showed approximately one vector genome copy per diploid genome. Neutralizing antibody titers against AAV increased after vector administration in all three subjects. No antibodies against factor IX were detected by either Western blot to detect non-neutralizing antibodies or coagulation assay to detect neutralizing antibodies. Despite direct administration into muscle, there was evidence for transient dissemination of virus. Vector sequences were detected in serum in all three subjects at 24 and 48 h, in saliva in all three subjects at 24 h, and in urine in one subject at 24 h after injection. There were no detectable vector sequences in semen at any time. One of the three subjects exhibited increased plasma levels of factor IX on multiple occasions beginning approximately eight weeks after vector administration. The highest level was 1.6% at week 10 and increased levels were detected out to at least 22 weeks. A second subject had levels up to 0.8%. Both subjects had reduced requirement for replacement factor. At the last interim report in December 2000, a total of eight subjects

were enrolled in the trial, three at 2×10^{11}, three at 5×10^{11}, and two at 2×10^{12} (19). No inhibitory or non-inhibitory antibodies to factor IX have been observed. One subject developed thrombocytopenia after vector administration, but the individual had a history of thrombocytopenia. No other toxicity was observed.

The results in this on-going study indicate that AAV-CMV-factor IX directed at muscle is safe with no evidence for inhibitor formation or germline transmission. Low levels of circulating factor IX were detected in some subjects.

Genstar trial in hemophilia A. The Genstar trial was approved by the Food and Drug Administration on March 9, 2000. This is a phase I trial in severe hemophilia A using MiniAdFVIII, a "gutless" adenovirus vector derived from type 5 adenovirus (Ad5) (20). MiniAdFVIII is devoid of all viral genes except essential cis elements and carries a 27 kB expression cassette that contains the full-length factor VIII cDNA under the control of the human 12.5 kB albumin promoter. Vector production is accomplished through a three-part packaging system that consists of the MiniAdFVIII, an ancillary Ad vector designed to support replication of the miniAd genome, and an AdE1 complementing cell line (A549E1) derived from A549 lung carcinoma cells. There are several novel features that the vector brings to this trial. It is the first clinical trial of any type using a "gutless" adenovirus. First generation adenoviral vectors cause inflammation due to the immune response to adenoviral proteins. The preclinical studies with MiniAdFVIII indicate that there is greatly reduced target organ toxicity compared to first generation adenoviral vectors (21). This is also the first clinical trial in hemophilia in which clotting factor expression is genetically targeted to a tissue, the liver. Another advantage of the "gutless" vector is the increased size of the expression cassette, which permits incorporation of sequences like the highly liver-specific albumin promoter. Restriction of expression to the liver should increase the efficiency of expression while reducing expression in antigen presenting cells, further reducing the immune response.

The MiniAdFVIII has been examined in several models. Initial studies in mice showed high level expression of factor VIII (21). Interestingly, although adenoviral vectors are non-integrating, expression of factor VIII was observed for up to a year from a single intravenous infusion. Mouse liver enzymes were maintained within normal limits following administration of MiniAdFVIII. In subsequent studies, cynomolgous monkeys were administered 4.3×10^{11}, 1.4×10^{12}, and 4.3×10^{12} vp/kg (22). Expression of functional FVIII in primate plasma was determined using a novel "capture" assay combined with a chromogenic assay. At all three vector doses, FVIII was detected at levels ranging from 28 to 88 mU/ml. In the highest dose group, thrombocytopenia and minimal elevation in liver transaminase levels were transient and similar to those previously observed in mice. No significant adverse effects were detected in the two lower dosage groups that resulted in the expression of therapeutic hFVIII levels.

Avigen liver-directed trial in hemophilia B. A proposed phase I, single dose, dose escalation trial of liver-directed approach in hemophilia B (23) has been approved by the Food and Drug Administration. The AAV vector is similar to that used in the on-going muscle trial but contains the α1-antitrypsin promoter and apolipoprotein E enhancer/hepatic control locus region to drive factor IX expression. Vector will be injected directly in the liver through a hepatic artery approach. In preclinical studies in rats, rAAV transduction levels in the liver and expression of human factor IX were 2-5-fold increased after portal vein or hepatic artery injection compared to peripheral tail vein injection.

Current Issues and Directions in Gene Therapy

These initial studies demonstrate that gene transfer in hemophilia A and B is safe and well tolerated in the approaches and doses used. No evidence for inhibitor formation has been demonstrated even though there were concerns about inhibitor development based on some of the results from animal models (16, 24, 25). Other than the single PCR positive result on one semen sample in the Chiron study, there were no serious adverse events attributed to treatment.

Importantly, all four clinical trials also demonstrated some degree of clinical efficacy. Increased levels of clotting factor, generally defined as measurable factor at least five days removed from treatment with exogenous factor, were observed in some subjects in each trial (Table 1). In each case, the levels of factor VIII or IX expression were low and variable. In these individuals, and even in some individuals in whom there was no detectable increase in factor expression, there was also a reduction in the requirement for treatment with exogenous clotting factor. Based on the results to date, the goal now is to increase expression without increasing the risk of toxicity.

One direction is to increase the dose of the available agents. In the Avigen preclinical studies, there was a clear dose-response between 1.3×10^{11} and 8.5×10^{12} vp/kg in hemophilic dogs (16). This suggests that as this trial moves to doses in the range of 2×10^{12} vp/kg and higher, increased levels of expression might be anticipated. Interestingly, there was not a clear dose-response in the Chiron trial. Over a log and half vector dose, from 2.2×10^{7} to 8.8×10^{8} TU/kg, there was no clear increase in transgene expression. Nevertheless, the lack of a predictable dose-response may simply indicate that the current studies are at a threshold dose. While the safety profile in the current trials supports an increase in dose, the Gelsinger experience warns against such increases without clear definition of a therapeutic window in animals.

Advances in vector development continue and promise to improve the efficiency and safety of gene transfer. One of the drawbacks with retroviruses has been their inability to infection in non-dividing cells. The generation of retroviral packaging and producer cell lines which permitted large-scale production of high titer retrovirus by Chiron (26) was an important early breakthrough which permitted the clinical trial in hemophilia to move forward. Nevertheless, the inability to infect non-dividing cells remains a problem and may explain the poor dose-response in the Chiron trial. Studies are currently underway in several laboratories to examine the use of hepatic growth factors to increase the number of dividing cells in the liver and thereby increase retrovirus uptake (27-29). The other area of intense investigation in the retrovirus field is the use of other retroviruses in the lentivirus family, including HIV, equine infectious anemia virus, feline immunodeficiency virus (FIV), and human foamy virus (30-35). These vectors retain some of the attractive features of the Moloney based retroviral vectors, such as stable integration into the host chromosome and targeted cellular uptake through coat proteins. They are able to infect non-dividing as well as dividing cells, although the efficiency of this has been recently questioned (36).

There are a number of exciting developments in the AAV field that will impact gene transfer in hemophilia. First, AAV serotype has been shown to have a dramatic effect on levels of transgene expression. Chao and coworkers have shown that, while the time to onset of detectable serum levels in a canine model of hemophilia B appeared the same for all serotypes, types 1, 3, and 5 produced 100- to 1000-fold more factor IX in muscle than type 2 (37). Twelve weeks after transduction, type 1 AAV continued to express levels of factor IX on average at 80 μg/ml

followed by type 5 (6.52 μg/ml), type 3 (3.27 μg/ml), type 4 (258 ng/ml), and type 2 (90 ng/ml). Second, different myofiber types (slow vs. fast) have different receptivity to AAV. Thus, AAV shows a preference for slow myofibers that correlates with expression of heparan sulfate proteoglycan receptors (38). Third, studies continue to examine the mechanism of site specific integration of AAV. The site of AAV integration in chromosome 19 (19q13.3-qter) is at a locus designated AAVS1. AAVS1 is closely linked to the slow skeletal troponin T gene, TNNT1 (39), raising an interesting correlate with the preference of AAV for slow myofibers. A better understanding of the mechanism of integration may lead to general methods for targeting integration, which would reduce the risk of insertional mutagenesis. Fourth, AAV appears to be less immunogenic than other vectors, especially first generation adenoviral vectors. Fields and coworkers found that activation of the immune system in response to AAV-mediated gene transfer is primarily through MHC class II mechanisms and is therefore more comparable to responses to infused proteins (40). In contrast, adenovirus-mediated gene transfer leads to activation through both MHC class I and II mechanisms and generates a more severe response that may include the transgene.

In general, adenoviral vectors have generated the highest levels of transgene expression and have been associated with the most severe toxicity, primarily in the liver. Studies to modify the immunogenicity of adenoviral vectors have resulted in the development of 'gutless' adenoviral vectors, vectors in which all of the adenoviral coding sequences have been removed (41, 42). These 'gutless' vectors have several potential advantages over previous generation adenoviral vectors. First, and perhaps most important, there are no viral proteins to be expressed, reducing the cellular immune response. This reduction in inflammation may be especially important in reducing hepatic toxicity (21, 43, 44). Second, by removing viral sequences, larger inserts can be accommodated, including tissue specific promoters and enhancers that can further increase the efficiency and specificity of transgene expression. The use of liver specific promoters has been shown to reduce the immune response to adenovirus (45), perhaps by preventing expression in antigen presenting cells. Another important advance has been the identification of CAR (*C*oxsackie virus and *A*denovirus *R*eceptor) and heparan sulfate glycosaminoglycans as cellular receptors for adenovirus (46, 47) and the identification of sequences in the adenovirus fiber knob that mediate the interaction with CAR (48, 49).

Conclusion

In summary, the initial trials of gene therapy in hemophilia A and B indicate that gene transfer is safe in the doses and routes of administration used. Low levels of expression of clotting factor have been observed in all of the clinical trials and the expression of clotting factor has been associated with clinical improvement. The challenge now is to increase clotting factor expression without increasing toxicity. As pointed out by Verma (50), focused efforts to better understand the basic biology of gene therapy is critical to continued advances and to the further development of gene transfer as a treatment for hemophilia and other inborn genetic disorders. These four clinical trials are our first important, halting steps in the process.

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Am J Pharmacogenomics 2001; 1 (2): 137-144
1175-2203/01/0002-0137/$22.00/0

Gene Therapy for Hereditary Hematological Disorders

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Contents

Abstract

The year 2000 saw the first successful treatment of a genetic disorder by gene therapy. Pediatric patients with X-linked severe combined immunodeficiency disorder (SCID-X1) received autologous CD34+ hematopoietic cells following *ex vivo* gene transfer using a retroviral vector, with subsequent demonstration of improved immune responses. A number of preclinical and clinical studies have been conducted with the aim of developing gene therapy for hemophilia, Fanconi anemia, sickle cell disease, β-thalassemia, chronic granulomatous disease, and other inherited hematological disorders. The greatest advances in novel approaches toward treatment of hematological disorders have been made in hemophilia, with 3 current phase I clinical trials ongoing. Two trials are investigating the safety and feasibility of utilizing either an *ex vivo*, non-viral gene transfer technique or an intravenous infusion of a retroviral vector to treat adults with severe hemophilia A (factor VIII deficiency). The third study involves intramuscular administration of an adeno-associated viral (AAV) vector for expression of factor IX in adult patients with hemophilia B. Results from this study and from preclinical studies preceding the trial demonstrate that it is possible to safely administer high doses of a viral vector *in vivo*.

1. Recent Success in Treatment of Severe Combined Immunodeficiency Disorder

The field of gene therapy has recently shown clinical success in a trial for infants with SCID-X1 severe combined immunodeficiency. These children, lacking the common gamma chain γc of the cytokine receptors for interleukin-2, -4, -7, and -15, received autologous CD34+ bone marrow cells transduced *ex vivo* with a retroviral vector for expression of functional γc cytokine receptor subunit. Treatment was carried out when the infants were less than 1 year old, and resulted in complete correction of the immunodeficiency in 4 of the 5 patients treated thus far.[1,2] There was complete restoration of functional B, T, and natural killer cell populations, and patients were successfully vaccinated, thus demonstrating proper antigen-specific immune responses. The first patient was enrolled in the trial 1.5 years ago, and is still showing evidence of a normal immune system. This study represents the first clearcut treatment of a genetic disorder by gene therapy.

Interestingly, the first gene therapy trial initiated a decade ago also involved a pediatric patient. In this pioneering experiment, a child with adenosine deaminase (ADA) deficiency was treated with autologous T cells transduced *ex vivo* with a retro-

viral vector for expression of functional ADA.[3] Correction of the immunodeficient phenotype was unclear, since the child remained on conventional treatment with PEG-ADA. However, in a later trial attempting to restore ADA activity in 3 infants by infusion of retrovirally transduced hematopoietic stem cells, treatment was clearly not successful, and the numbers of engrafted cells were low.[4] The success of the recent SCID-X1 study may be due to several factors, such as the use of fibronectin fragment CH296 for enhanced retroviral gene transfer to hematopoietic stem cells and progenitor cells, and a greater selective advantage of γc-expressing cells than was the case for ADA-expressing cells in the earlier trials.[5]

2. Gene Therapy for Hemophilia

Perhaps the most continual steady advances in gene therapy for hematological disorders have been made in treatment of hemophilia.[6] There are currently 3 phase I safety trials being carried out in gene therapy for hemophilia; 2 for factor VIII (F.VIII) deficiency (hemophilia A) and one for factor IX (F.IX) deficiency (hemophilia B). These X-linked coagulation disorders result in a severe phenotype for patients with factor activity of <1% of normal, characterized by frequent spontaneous bleeding into joints and soft tissue and the risk of fatal intracranial or retroperitoneal bleeding. Years of experience with prophylactic infusion of clotting factor concentrates have shown that maintaining factor levels at >1% of normal activity markedly ameliorates the phenotype of the disease. The minimal goal of a gene therapy approach, therefore, has to be continuous systemic supply of coagulation factor by the donated gene at levels >1% of normal. The general concept of *ex vivo* and *in vivo* gene transfer strategies is outlined in figure 1.

Normal synthesis of clotting factor molecules is characterized by extensive post-translational modifications including glycosylation, proteolytic cleavage, γ-carboxylation (in the case of factor IX), and others. Furthermore, factor VIII has to be stabilized by von Willebrand factor following secretion. Interestingly, although both are normally synthesized in the liver, functional factor VIII and factor IX can be produced by a variety of cell types. This observation is reflected in the design of the current clinical trials. While the benefit of a continuous supply of factor VIII or factor IX to pediatric patients with severe hemophilia is obvious, these initial safety and even subsequent efficacy trials will be confined to adult patients. Given the novelty of the approaches and the complicated evaluation of risks resulting from stable changes of the genetic makeup of somatic tissue, it is not surprising from an ethical standpoint

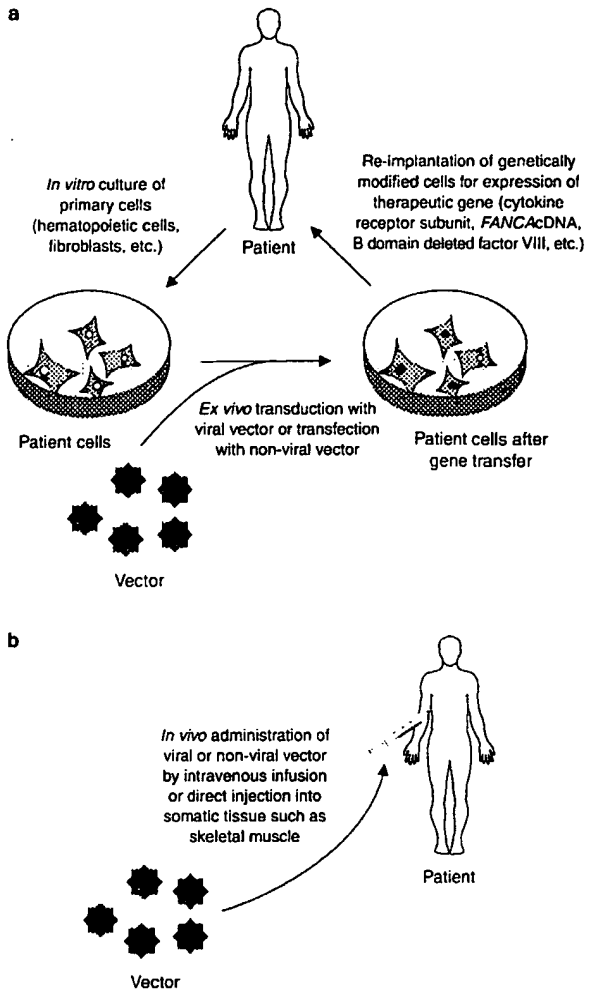

Fig. 1. Gene transfer by *ex vivo* or *in vivo* transduction/transfection of target cells. **(a)** *Ex vivo* method: Primary cells obtained from the patient from bone marrow, peripheral blood, or by tissue biopsy are cultured *in vitro* and subsequently transduced with a viral vector or transfected with a non-viral vector such as plasmid DNA. Following gene transfer, cells are reimplanted into the patient. **(b)** *In vivo* method: The vector is directly administered to the patient, e.g. by intravenous or intramuscular injection.

that children are not considered appropriate candidates for initial gene transfer studies.

Two of the hemophilia trials focus on treatment of factor VIII deficiency. One study, sponsored by the Chiron Corporation, is based on intravenous infusion of a highly concentrated retroviral vector pseudotyped with an amphotropic envelope for expression of B domain-deleted factor VIII.[7] In a preclinical study published by a different group, therapeutic levels of human factor VIII expression were demonstrated for a retroviral vector infused intravenously in neonatal mice.[8] However,

in these animals, hepatocytes are actively dividing, which is a general requirement for retroviral transduction.

Another trial, sponsored by Transkaryotic Therapies and the Beth Israel Deaconess Medical Center in Boston, is based on *ex vivo* transfection of autologous fibroblasts obtained from a skin biopsy from the patients.[9] These cells undergo stable transfection with a plasmid expressing B domain-deleted factor VIII, and are then clonally expanded. Fibroblasts expanded from one factor VIII-expressing clone are implanted into the omentum of the patient (up to 4×10^8 cells). Expression of factor VIII at levels >1% of normal in several patients, albeit transient, has been reported at the annual meeting of the American Society of Hematology (2000).[9] Details on the scientific background of both of these trials (in the form of published preclinical data) are currently not available.

2.1 Muscle-Directed Viral Gene Transfer for Treatment of Hemophilia B

To date, the only clinical study on hemophilia B (a collaboration between Avigen, Inc., The Children's Hospital of Philadelphia, and Stanford University) takes advantage of the ability of adeno-associated (AAV) vectors to efficiently transfer genes to non-dividing cells and the ability of muscle fibres to produce biologically active factor IX.[10,11] AAV is a non-pathogenic, replication-deficient virus with a 4.7-kb single-stranded DNA genome. In the vector, which is produced in a helper virus-free system by transient transfection of human embryonic kidney 293 (HEK-293) cells, all viral coding sequences are removed and replaced by an expression cassette for factor IX.[12,13] In published preclinical studies, direct injection of such a vector into multiple intramuscular (IM) sites at a single time point resulted in secretion of factor IX from transduced skeletal muscle fibres and vector dose-dependent expression of up to 7% of normal human factor IX levels in mice and up to 1.4% in hemophilia B dogs (using a canine factor IX transgene).[14-16] These experiments utilized a strong viral promoter, CMV I.E. (cytomegalovirus immediate early) enhancer/promoter, for expression of factor IX. Systemic expression in hemophilic dogs has been sustained for >3 years (Herzog, Nichols, High, unpublished results). Expression levels for a given dose of vector/kg were 3- to 5-fold lower than predicted from mouse studies for the 200-1000-fold scale-up to the large animal model. Figure 2 outlines an optimal design for preclinical studies intended to establish a novel approach toward gene therapy for hemophilia. Demonstration of efficacy in an animal model of hemophilia B and the excellent toxicity profile of the vector (AAV-CMV-hF.IX, Coagulin-B™)[1] facilitated its introduction to clinical application.[17]

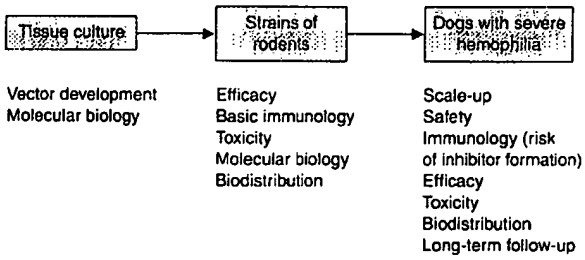

Fig. 2. A strategy for development of preclinical data to establish a novel approach for the treatment of hemophilia by gene therapy. At first, a vector is tested for efficacy in mouse models of gene transfer of factor VIII or factor IX. Once it is established that the vector directs stable therapeutic levels of expression without serious toxicity or inflammatory responses, the approach is evaluated in a canine model of hemophilia using the available canine cDNAs for factor VIII or factor IX. This strategy permits evaluation of efficacy, scale-up, risk of inhibitor formation, duration of expression, toxicity, and long-term follow-up in a large animal model with severe disease. Because of the size, life expectancy, and well-characterized phenotype of the canine hemophilia strains, they are invaluable for conducting such studies.

In vivo administration of large amounts of a viral vector to patients with a prolonged life expectancy raises a new set of issues previously not encountered in gene therapy. An obvious advantage of the AAV vector is the vector's reduced potential for causing inflammatory or cellular immune responses.[18] A second important issue raised by the regulatory agencies is the risk of inadvertent germ line transmission of vector sequences, which is of particular relevance for vectors with the capability of integrating into chromosomal DNA of transduced cells.[19,20] Arruda et al. have described in a study on intramuscular administration of AAV vector in rabbits that, despite hematogenous dissemination, no vector sequences were detected in semen samples.[21] A detailed analysis of these findings is currently being carried out. Another important concern for any new treatment of hemophilia is the formation of inhibitory antibodies against factor VIII or factor IX, which may complicate conventional treatment. Some insight into the complicated topic of immune responses against a secreted transgene product can be obtained from studies in an animal model of hemophilia with a vector expressing a species-specific transgene. A published study on intramuscular injection of the AAV-canine factor IX vector in hemophilia B dogs with a factor IX missense mutation (resulting in the absence of circulating factor IX antigen) included the observation of a transient inhibitor during the first 2 months after vector administration in 1 of the 5 treated dogs.[16] Continued studies on the immunology of AAV-mediated gene transfer of coagulation factors, as well as improved vector systems utilizing novel enhancer/promoter com-

1 Use of tradenames is for identification purposes only and does not imply endorsement.

binations and different serotypes of AAV will likely further improve the safety and efficacy of the gene transfer protocol.[18,22,23]

In 1999, a phase I trial of intramuscular administration of an AAV-factor IX vector to patients with severe hemophilia B (<1% factor IX activity) was initiated. The trial was designed as a dose escalation study with 3 patients per dose cohort, with a starting dose of 2×10^{11} vector genomes/kg bodyweight. At a single time point, patients were injected at multiple skeletal muscle sites using ultrasound guidance to minimize vascular dissemination of vector. Table I summarizes some of the major study goals of a phase I clinical trial that is based on *in vivo* delivery of a viral vector to patients with hemophilia. A preliminary report on the 3 patients in the lowest dose cohort gave clear evidence of gene transfer and expression in injected muscle tissue obtained by biopsy.[24] DNA analysis and immunostaining of muscle sections showed transfer and expression of the vector-encoded factor IX gene. Patients had no signs of local or systemic toxicity, and also did not develop antibodies against factor IX. DNA analysis of semen samples gave no evidence for germ line transmission. These data are encouraging for the efficacy and safety of the vector system, and an evaluation of patients treated with higher doses of vector is currently underway. The goal of a subsequent phase II clinical trial will be defining a vector dose that can reliably raise factor IX levels above 1% of normal.

Table I. Safety assessment in a phase I clinical trial for treatment of hemophilia with a viral vector

Safety of the procedure
Vector-related toxicity
Risk of inhibitor formation
Biodistribution/germ line transmission
Evidence for gene transfer and expression
Effect of pre-existing immunity (neutralizing antibodies against vector particles)
Long-term follow-up (including assessment of risks associated with the possibility of insertional mutagenesis)

2.2 Liver-Directed Viral Gene Transfer for Treatment of Hemophilia A and B

A second protocol for treatment of hemophilia B by *in vivo* delivery of an AAV vector has been developed in parallel with the muscle-directed approach. Infusion of an AAV into the portal circulation results in transduction of up to 5% of hepatocytes with a strong tropism of the vector to the liver.[25] This procedure, although more invasive than the muscle protocol, has the potential to be curative because higher levels of circulating factor IX per delivered viral particle can be obtained.[26] Thus, complete correction of the hemophilia B phenotype has been achieved in hemophilia B mice.[27] Extensive safety studies with regard to toxicity, reproductive toxicology, and immunogenicity have also been very favourable for this approach, and a phase I clinical trial has now been approved.[28] Success of a liver-directed protocol may be affected by the relatively high prevalence of neutralizing antibodies against AAV particles in the serum of patients (pre-existing immunity) or by the presence of hepatitis C, which has a high prevalence in the adult hemophilia population (transmitted through plasma-derived concentrates). Whether hepatitis may influence the risk of an immune response to the transgene product in liver-directed gene therapy is unknown, partially because of the absence of relevant animal models. Treatment of hemophilia A by liver-directed gene therapy with an AAV vector has been the subject of recent investigation in the field, and a vector that may be suitable for this task has been developed.[29] However, rapid development of such a vector has been hampered by the size of the factor VIII cDNA and the narrow packaging limit of AAV. Another study has shown that this limitation can be overcome by infusion of two AAV vectors expressing the heavy and light chains of factor VIII separately.[30]

Development of a protocol for adenoviral-mediated gene transfer of coagulation factors has been complicated by the immunogenicity of the vector. To create adenoviral vectors with reduced immunogenicity, so-called gutted adenoviral vectors devoid of any viral coding sequences were developed.[31] These vectors can accommodate large inserts and can therefore be used to express full-length factor VIII. Intravenous infusion of such a vector has been shown to direct super-physiological levels of human factor VIII in hemophilia A mice from a liver-specific albumin promoter.[32] The vector is now approved for a phase I clinical trial. Although the vector is devoid of viral coding sequences, there is more recent evidence for acute phase immune responses against adenovirus (resulting in hepatotoxicity and potentially systemic inflammatory responses) that may be independent of gene expression, i.e. they are caused by the viral particles themselves.[33,34] Such responses remain a safety concern even for gutted adenoviral vectors. Furthermore, recent studies also describe a threshold effect for transduction of hepatocytes with adenoviral vector, which raises the question of whether toxicity data obtained at low vector doses (i.e.below this threshold) will be useful in predicting toxicity at higher doses.[35] Hepatotoxicity and inhibitor formation have been reported following adenoviral gene transfer of canine factor VIII to the liver of hemophilia A dogs. This set of experiments, however, did not utilize a completely 'gutted' vector.[36]

3. Fanconi Anemia

Fanconi anemia (FA) is an autosomal recessive disorder associated with susceptibility to cancer and progressive bone marrow failure. Other clinical manifestations include growth retardation, pigmented skin lesions and the presence of multiple anomalies most commonly involving the genitourinary urinary tract and the extremities.[37] Complications of pancytopenia secondary to bone marrow failure contribute significantly to the morbidity and mortality of FA. However, as treatment for bone marrow failure improves, both hematological and solid tumor malignancies, with myeloid leukemia the most common, are becoming an increasing problem for patients with FA; malignancy occurs in 20% to 50% of patients. The diagnostic test for FA is the diepoxybutane chromosomal fragility test.[38] A cardinal feature of FA is the profound number of chromosomal breaks that occur in the presence of bifunctional alkylating agents such as diepoxybutane or mitomycin C.

The range of the underlying molecular defects in FA is broad, not surprising given the long recognized breadth of clinical manifestations.[39] There are 7 complementation groups that have been well described, designated A to G. The genes for groups A, C, D, E, F and G have been cloned; group A accounts for 66% of cases, group C for 12% and groups D, E, F and G for <2% each.[40] The first FA gene to be cloned was *FANCC* in 1992 followed by the *FANCA* gene in 1996.[41,42] Gene-based therapies are being developed for both groups A and C, and much of what is being learned is likely to be applicable to the other groups.

Because most of the serious and earlier manifestations of FA involve aberrant proliferation or malignant transformation of hematopoietic stem cells, gene therapy using *ex vivo* gene transfer into hematopoietic stem cells is an attractive treatment option. If the defect that leads to early stem cell death or malignant transformation can be reversed in a sufficient number of hematopoietic stem cells that subsequently have a survival and proliferative advantage, then aplastic anemia can be avoided and the risk of myeloid leukemia lessened.

A number of groups have been investigating the use of gene transfer in both diagnosis and treatment of FA.[43]. *Ex vivo* gene transfer using retroviral vectors can be used to determine the complementation group of newly diagnosed patients.[44] The current approach to gene therapy for FA is *ex vivo* transduction of the normal *FANCC* gene into autologous hematopoietic stem cells using a retroviral vector.[45] Murine models of FA have been developed which are aiding in the development of gene therapy strategies.[46] An interesting observation in murine experiments has been the *in vivo* selection for corrected hematopoietic stem cells, presumably because of their survival advantage.[47]

The first clinical trial of gene transfer for the treatment of FA was performed at the National Institutes of Health (NIH). Four patients with FA-C underwent 1 to 4 cycles of gene transfer, with each cycle consisting of 1 to 2 infusions of autologous hematopoietic stem cells (HSCs) transduced with a retroviral vector expressing the *FANCC* gene.[48] Although the overall response was minimal, the trial demonstrated the feasibility of gene therapy for FA. A trial involving a similar strategy is currently underway at the University of North Carolina at Chapel Hill with *FANCA* as the therapeutic gene. A retroviral vector containing the *FANCA* cDNA is used to transduce CD34+ cells harvested from the bone marrow of patients with FA-A. The treated cells are then infused intravenously back into the patient without any preconditioning of the bone marrow. Thus far, 4 patients have entered the trial, and have tolerated the procedure well.[49] These early experiences with gene therapy for FA have illustrated the limitations of harvesting CD34+ cells from patients with progressive bone marrow failure. Improved harvesting techniques and utilizing *in vitro* expansion will assist in overcoming this limitation. As with allogeneic bone marrow transplant for FA, the use of autologous HSCs as a target for gene therapy does not eliminate the risk of solid tumor malignancies. Nonetheless, successful gene therapy using autologous HSCs would be a significant improvement over the current therapeutic options for FA. The chief complications of allogeneic bone marrow transplant are avoided using the above gene therapy strategy.

4. Chronic Granulomatous Disease

Chronic granulomatous disease (CGD) is an inherited disorder associated with defective phagocytic 'repiratory burst' activity. As a result of this impaired phagocytic function, CGD patients experience recurrent bacterial and fungal infections and have abnormal inflammatory responses, which lead to granuloma formation and poor wound healing. CGD affects approximately 1 in 200 000 persons. The diagnosis of CGD is made using the nitroblue tetrazolium (NBT) method, which relies on the intracellular reduction of NBT by superoxide anion to a blue formozan precipitate that can be seen microscopically.[50] The underlying molecular defects that lead to CGD have recently been elucidated, making gene therapy a potential treatment modality. The majority of cases of CGD arise from defects in either the X-linked $gp91^{phox}$ gene, accounting for approximately two-thirds of cases, or the autosomal $p22^{phox}$ gene, accounting for most of the remaining cases.[51]

CGD is an excellent candidate disease for *ex vivo* gene therapy involving hematopoietic stem cells, with the goal being to establish a population of myeloid precursors containing the normal

gene that would generate a sufficient number of normally functioning peripheral phagocytes.[52] If a stable population could not be established, it is conceivable that periodic infusions of treated autologous myeloid precursors could result in a therapeutic response.[53] In mouse models of CGD, genetic correction of NADPH oxidase activity has been achieved *in vivo* with stem cell gene therapy, and the treated mice have a decreased risk of infection.[54,55] A limitation to this approach is the conditioning regimen that would be needed to allow for sufficient engraftment of the transduced HSCs. It is possible that non-myeloablative conditioning regimens could be used in order to reduce the toxicity associated with conventional regimens.

5. Hemoglobinopathies

The hemoglobinopathies, which include the thalassemias and the sickle cell anemias, affect a large number of people throughout the world. Therefore, the development of an effective gene therapy approach to treating these common hemoglobinopathies would have an enormous impact. Unfortunately, gene transfer strategies for hemoglobinopathies must overcome a number of obstacles.

As the understanding of the molecular biology behind globin gene expression has increased, both new challenges and new possibilities have arisen. The erythrocyte-progenitor specific expression of the β-, δ-, and γ-globin genes is tightly regulated by a locus control region. Poorly regulated expression could result in an imbalance in the production of β-globin versus α-globin chains, resulting in dyserythropoiesis and shortened red blood cell survival. Erythrocyte-progenitor specific expression is required because globin gene expression would be disruptive to non-erythropoietic hematopoietic cells. Therefore, the challenge for globin gene addition strategies has been to confine the expression of the transgene to erythropoietic cells, and achieve a sustained, controlled high level of globin chain production.

For sickle cell disease, the simple insertion of a normal β-globin gene into hematopoietic stem cells is unlikely to result in full therapeutic effect unless production of sickle β-globin is reduced. Fortunately, other potential strategies exist. An approach that would result in increased γ-globin (fetal hemoglobin) production would result in some therapeutic response.[56] Methodologies based on homologous recombination have been used in an attempt to replace the abnormal portion of the gene with normal sequences. For sickle cell disease, correction of the A-to-T point mutation within the sixth codon of the β-globin gene using RNA-DNA oligonucleotide-targeted mutagenesis has been accomplished in the laboratory.[57] However, the efficiency and clinical utility of such a strategy is unclear. An alternative approach is based on RNA repair using ribozymes.[58]

Fortunately, the technologies for harvesting, purifying, processing, expanding and transplanting autologous hematopoietic stem cells has advanced considerably over the last decade, making gene therapy involving this cellular compartment more readily accomplished. Recent improvements in vector and gene transfer technology have improved transduction efficiency of hematopoietic stem cells, e.g. by vectors with a pseudotyped envelope.[59] However, vector development for globin gene transfer has lagged behind other hematological diseases. The globin genes and their locus control region have proved difficult to package into retroviral vectors, which have been the vectors of choice for *ex vivo* hematopoietic stem cell gene therapy. Moreover, cryptic splice sites may have been responsible for frequent deletions in the transferred β-globin gene.[60] Finally, the expression of the integrated transgene tends to decrease over time partly due to position effects and gene silencing.[60]

Efforts have been ongoing to increase and stabilize expression of β-globin.[61,62] A breakthrough discovery has been made recently with the use of a lentiviral vector for efficient transfer of a human β-globin gene to hematopoietic stem cells of β-thalassemic mice, resulting in therapeutic levels of expression with substantial increases in red blood cell counts and hemoglobin levels.[63] The HIV-based vector was superior in packaging capacity of genomic globin sequences, suppression of unwanted splicing of the viral RNA genome, gene transfer to stem cells, and reduced position effect and gene silencing of integrated vector sequences, when compared with studies using more traditional retroviral vectors.[63,64] These results have a profound impact on the field and may make gene therapy for thalassemia (and perhaps other hemoglobinopathies) a realistic goal for the not-so-distant future.

6. Conclusion

In the past 2 years, numerous milestones in the pursuit of gene therapy for inherited genetic defects were reached. Multiple ground-breaking studies were published exploring the treatment of immune deficiencies, hemophilia A and B, and hemoglobinopathies using gene therapy techniques were published. The first unequivocal success in treatment by gene transfer has been achieved for patients with a particular form of SCID. The safety of several novel treatment approaches toward hemophilia, using viral and non-viral as well as *in vivo* and *ex vivo* protocols, are currently being carried out. These studies will enable the assessment of safety and feasibility of a number of vector-target tissue combinations for human gene therapy, thus making gene-

based strategies a realistic alternative for the treatment of hematological and other disorders.

Acknowledgements

R.W. Herzog is supported by a Career Development Award by the National Hemophilia Foundation. The authors thank Dr. K.A. High for suggestions for the manuscript.

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Article title	Gene therapy in vascular medicine: Recent advances and future perspectives
Article identifier	0163725801000535
Authors	Morishita_R Aoki_M Kaneda_Y Ogihara_T
Journal title	Pharmacology and Therapeutics
ISSN	0163-7258
Publisher	Elsevier USA
Year of publication	2001
Volume	91
Issue	2
Supplement	0
Page range	105-114
Number of pages	10
User name	Adonis
Cost centre	
PCC	$22.00
Date and time	Tuesday, November 19, 2002 9:06:06 PM

ELSEVIER

Pharmacology & Therapeutics 91 (2001) 105–114

Pharmacology & Therapeutics

Associate editor: M. Endoh

Gene therapy in vascular medicine: recent advances and future perspectives

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Abstract

Gene therapy is emerging as a potential strategy for the treatment of cardiovascular diseases, such as restenosis after angioplasty, vascular bypass graft occlusion, and transplant coronary vasculopathy, for which no known effective therapy exists. The first human trial in cardiovascular disease was started in 1994 to treat peripheral vascular disease using vascular endothelial growth factor. In addition, therapeutic angiogenesis using the vascular endothelial growth factor gene was applied in the treatment of ischemic heart disease. The results from these clinical trials seem to exceed expectation. Improvement of clinical symptoms in peripheral arterial disease and ischemic heart disease has been reported. At least five different potent angiogenic growth factors have been tested in clinical trials to treat peripheral arterial disease or ischemic heart disease. In addition, another strategy for combating disease processes, to target the transcriptional process, has been tested in a human trial. Transfection of cis-element double-stranded oligodeoxynucleotides is an especially powerful tool in a new class of antigen strategies for gene therapy. Transfection of double-stranded oligodeoxynucleotides corresponding to the cis sequence will result in the attenuation of the authentic cis-trans interaction, leading to the removal of trans-factors from the endogenous cis-elements, with subsequent modulation of gene expression. Genetically modified vein grafts transfected with a decoy against E2F, an essential transcription factor in cell cycle progression, revealed apparent long-term potency in human patients. This review focuses on the future potential of gene therapy for the treatment of cardiovascular disease.

Keywords: cis-Element decoy; Antisense; Angiogenesis; VEGF; Restenosis

Abbreviations: CAD, coronary artery disease; decoy, cis-element double-stranded oligodeoxynucleotides; FGF, basic fibroblast growth factor; HGF, hepatocyte growth factor; NF-κB, nuclear factor-κB; ODN, oligodeoxynucleotide; PAD, peripheral arterial disease; PCNA, proliferating-cell nuclear antigen; VEGF, vascular endothelial growth factor; VSMC, vascular smooth muscle cell.

Contents

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0163-7258/01/$ – see front matter
PII: S0163-7258(01)00150-4

1. Introduction

Somatic gene therapy consists of the introduction of normal genes into the somatic cells of patients in order to correct an inherited or acquired disorder through the synthesis of specific gene products in vivo. In general, there are three methods of gene modification: (1) gene replacement, (2) gene correction, and (3) gene augmentation. Gene augmentation is the most promising technique for the modification of targeted cells in the therapy of vascular disease. For this purpose, many in vivo gene transfer methods have been developed. In vivo gene transfer techniques for vascular applications include (1) viral gene transfer: retrovirus, adenovirus, adeno-associated virus, or hemagglutinating virus of Japan (Sendai virus); (2) liposomal gene transfer using cationic liposomes; and (3) naked plasmid DNA transfer. These in vivo gene transfer techniques have different advantages and disadvantages. Although current in vivo methods for vascular gene transfer are still limited by the lack of efficiency and potential toxicity, recent advances in in vivo gene transfer may provide the opportunity to treat vascular diseases such as peripheral arterial disease (PAD) by manipulating angiogenic growth factor genes. In this review, we will discuss the potential application of gene therapy for the treatment of vascular disease.

2. Gene therapy for vascular diseases

2.1. Gene therapy to treat peripheral arterial disease using therapeutic angiogenesis

Critical limb ischemia is estimated to develop in 500–1000 individuals per million per year (Second European Consensus Document on Chronic Critical Leg Ischemia, 1991). In a large proportion of these patients, the anatomical extent and the distribution of arterial occlusive disease make the patients unsuitable for operative or percutaneous revascularization. Thus, the disease frequently follows an inexorable downhill course (Dormandy et al., 1989; Rutherford et al., 1986). Of importance, there is no optimal medical therapy for critical limb ischemia, as the Consensus Document of the European Working Group on Critical Limb Ischemia concluded (Second European Consensus Document on Chronic Critical Leg Ischemia, 1991). Thus, in patients with critical limb ischemia, amputation, despite its associated morbidity, mortality, and functional implications (Second European Consensus Document on Chronic Critical Leg Ischemia, 1991; Most & Sinnock, 1983; Tyrrell & Wolfe, 1993), is often recommended as a solution to the disabling symptoms, in particular, excruciating ischemic rest pain (Tyrrell & Wolfe, 1993; Eneroth & Persson, 1992). Indeed, a second major amputation is required in nearly 10% of such patients. Consequently, the need for alternative treatment strategies in patients with critical limb ischemia is compelling. Therefore, novel therapeutic modalities are needed to treat these patients. In the pathophysiology of the disease, in the presence of obstruction of a major artery, blood flow to the ischemic tissue is often dependent on collateral vessels. When spontaneous development of collateral vessels is insufficient to allow normal perfusion of the tissue at risk, ischemia occurs. Recently, the efficacy of therapeutic angiogenesis using vascular endothelial growth factor (VEGF) gene transfer has been reported in patients with critical limb ischemia (Isner et al., 1996a, 1996b, 1998; Baumgartner et al., 1998). Thus, the strategy for therapeutic angiogenesis using angiogenic growth factors should be considered for the treatment of patients with critical limb ischemia. Most of the studies have used VEGF, also known as vascular permeability factor, as well as a secreted endothelial-cell mitogen. The endothelial cell specificity of VEGF has been considered to be an important advantage for therapeutic angiogenesis, as endothelial cells represent the critical cellular element responsible for new vessel formation.

A human clinical trial using the VEGF gene was started in 1994 by J. M. Isner at Tufts University (Boston, MA, USA). An initial trial was performed using a hydrogel catheter with naked $VEGF_{165}$ plasmid. Although this procedure seems to be effective in the stimulation of collateral vessel formation in patients with PAD (Isner et al., 1996a), it is not ideal for the treatment of many patients, as most patients lack an appropriate target vascular lesion for catheter delivery. Thus, his group applied intramuscular injection of naked plasmid DNA encoding the $VEGF_{165}$ gene. Exceeding expectation, this clinical trial demonstrated clinical efficacy for the treatment of PAD (Baumgartner et al., 1998; Isner et al., 1998) (Table 1). Since then, numerous angiogenic growth factors, such as $VEGF_{121}$, VEGF-2, and basic fibroblast growth factor (FGF), have been tested in clinical trials. In addition to intramuscular injection of naked plasmid DNA, adenoviral delivery of angiogenic growth factors was also utilized in these trials (Table 2), despite an unfortunate accident reported at the University of Pennsylvania (Philadelphia, PA, USA) last year (Marshall, 1999).

We also identified hepatocyte growth factor (HGF) as a novel candidate for therapeutic angiogenesis. HGF is a mesenchyme-derived pleiotropic factor that regulates cell growth, cell motility, and morphogenesis of various types of cells, and, thus, is considered a humoral mediator of epithelial-mesenchymal interactions responsible for morphogenic tissue interactions during embryonic development and organogenesis (Nakamura et al., 1989). Previously, we and others reported that HGF stimulated angiogenesis in a rabbit ischemic hind limb model (Morishita et al., 1999; Belle et al., 1998; Hayashi et al., 1999). Unexpectedly, the angiogenic activity of HGF was more potent than that of VEGF or basic FGF in vitro (Nakamura et al., 1996), as well as in vivo (Hayashi et al., 1999). Currently, we are carrying out a human clinical trial of gene therapy to treat PAD using the HGF gene. Although these trials have not been finished, gene therapy using angiogenic growth factors to treat PAD seems to be feasible in the near future.

Table 1
Results from human gene therapy

Reference	Gene (number of patients)	Disease	Institute	Dose (μg)	Vector	Adverse effects	Clinical outcome
Isner et al., 1996a	$pVEGF_{165}$ (n = 1)	ASO	Tufts	2000	Plasmid + hydrogel	Leg edema	Angiogensis (angiogram)
Baumgartner et al., 1998	$pVEGF_{165}$ (n = 10)	ASO	Tufts	2000	Intramuscular plasmid		Improvement in collateral formation (7 of 10 patients) by magnetic resonance angiography
Isner et al., 1998	$pVEGF_{165}$ (n = 6)	TAO	Tufts	2000–4000	Intramuscular plasmid		Improvement in ulcer (3 of 5 patients), disappearance of rest pain (2 of 7 patients), amputation (2 of 7 patients)
Kalka et al., 2000	$pVEGF_{165}$	ASO	Tufts		Intramuscular plasmid		Increase in endothelial progenitor cells
Baumgartner et al., 2000	$pVEGF_{165}$ (n = 62)	ASO	Tufts		Intramuscular plasmid	Leg edema (34%)	Leg edema can be controlled by oral antidiuretics
Losordo et al., 1998	$pVEGF_{165}$ (n = 5)	ICM	Tufts	125	Minithoractomy plasmid	Transient arrhythmia	Decrease in nitroglycerin, increase in blood flow (single-photon emission computed tomography and angiogram), no change in ejection fraction
Vale et al., 1999	pVEGF-2 (n = 1)	ICM	Tufts	6 place	Minithoractomy plasmid		Increase in blood flow [NOGA, Biosense-Webster Co. (Cordis, MD USA)]
Vale et al., 2000	$pVEGF_{165}$ (n = 13)	ICM	Tufts	250 or 500	Minithoractomy plasmid		Decrease in nitroglycerin tablets, increase in blood flow (NOGA, Biosense-Webster Co.)
Rosengart et al., 1999b	$pVEGF_{121}$ adenovirus (n = 21)	ICM	Cornell	400	Minithoractomy adenovirus	None	Improvement in treadmill exercise test after 30 days
Resengart et al., 1999a	$pVEGF_{121}$ adenovirus (n = 21)	ICM	Cornell	400	Minithoractomy adenovirus	None Anti-adenovirus antibody (-)	Not effective in bypass case Decrease in nitroglycerin tablets after 6 months (sole theraphy)
Laitinen et al., 2000	pVEGF (n = 10)	Restenosis	Kuopio	1000 μg/1000 μL	Cationic liposome + catheter	None	No change in restenosis rate
Mann et al., 1999	E2F decoy (n = 17)	Vein graft	Harvard	40 μM/L	Ex vivo oligo alone	None	Decrease in graft failure rate

ASO, atherosclerosis obleterance; ICM, ischemic myocardial infarction; TAO, thromboangitis obliterans.

Table 2
Clinical trial in gene therapy using angiogenic growth factors

Company	Vector/gene	Target disease
Vascular Genetics	Plasmid/VEGF-2	PAD, CAD
GenVec	Adenovirus/$VEGF_{121}$	PAD, CAD
Collateral Therapeutics	Adenovirus/FGF-4	CAD
Valentis	Plasmid/$VEGF_{165}$	PAD, CAD
Genzyme	Adenovirus/HIF-1α	PAD, CAD
Gencell	Plasmid/FGF-1	PAD

HIF, hypoxia-inducible factor.

A similar idea has been applied to treat coronary artery disease (CAD). A human gene therapy trial to treat CAD using the $VEGF_{165}$ gene has been started by Isner and colleagues (Losordo et al., 1998; Vale et al., 2000). His group performed intramuscular injection of naked plasmid DNA encoding the VEGF gene into the ischemic myocardium through a mini-operation. Similar to human trials in PAD, transfection of the VEGF gene resulted in a marked increase in blood flow and improved clinical symptoms without apparent toxicity (Losordo et al., 1998) (Table 1). More recently, the results from 13 consecutive patients with chronic stable angina have been reported (Vale et al., 2000). Although all of them had failed conventional therapy (drugs, percutaneous transluminal coronary angioplasty, and/or coronary artery bypass graft), reduction in the size of the defects documented by serial single-photon emission CT-sestamibi imaging was observed after direct myocardial injection of $phVEGF_{165}$ via a minithoracotomy (Vale et al., 2000). These data clearly suggest that $phVEGF_{165}$ gene therapy may successfully rescue foci of hibernating myocardium. Following this success, gene therapy using the $VEGF_{121}$ gene was performed by intramuscular injection of the adenoviral vector (Rosengart et al., 1999b). A Phase I study using adenovirus-mediated transfection of the $VEGF_{121}$ gene demonstrated clinical safety (Rosengart et al., 1999a). More recently, intracoronary infusion of adenovirus encoding the FGF gene was performed in a multi-center trial as Phase I/IIa. The report documented that intracoronary infusion of the FGF gene improved cardiac dysfunction without severe toxicity. Our group has also applied to start a human gene therapy protocol using intracardiac-muscular injection of HGF plasmid DNA through surgical operation, since overexpression of the HGF gene stimulated angiogenesis and increased blood flow in rat and canine myocardial infarction models (Aoki et al., 2000; Ueda et al., 1999). In summary, the treatment of CAD by therapeutic angiogenesis is feasible using gene therapy.

What is the advantage of gene therapy as compared with recombinant therapy? (1) It has the potential to maintain an optimally high and local concentration over time. This issue may be critical in the case of arterial gene therapy. However, in the case of therapeutic angiogenesis, it may be preferable to deliver a lower dose over a period of several days or more from an actively expressed transgene in the iliac artery rather than a single or multiple bolus doses of recombinant protein in order to avoid side effects. (2) Regarding economics, which therapy would ultimately cost more to develop, implement, and reimburse, particularly for those indications requiring multiple or even protracted treatment, needs to be considered. (3) The feasibility of a clinical trial using recombinant protein currently is limited by the lack of approved or available quantities of human quality grade recombinant protein, due in large part to the nearly prohibitive cost of scaling up from research to clinical grade protein. Based upon these properties, it is assumed that the first gene therapy drug may be commercially available by 2004.

2.2. Gene therapy for restenosis after angioplasty

Another important disease potentially amenable to gene therapy in cardiovascular disease is restenosis after angioplasty. Balloon angioplasty is one of the major therapeutic approaches to coronary artery stenosis. However, restenosis occurs in 30–40% of the patients after angioplasty (Gibbons & Dzau, 1994). Intimal hyperplasia develops in large part as a result of vascular smooth muscle cell (VSMC) proliferation and migration induced by a complex interaction of multiple growth factors that are activated by vascular "injury" (Gibbons & Dzau, 1994). The process of VSMC proliferation is dependent on the coordinated activation of a series of cell cycle regulatory genes that results in mitosis. Therefore, inhibition of the cell cycle using nonphosphorylated retinoblastoma gene or anti-oncogenes, such as p53 and p21, has been reported in several animal models (Chang et al., 1995a, 1995b; Yonemitsu et al., 1998) (Table 3). However, none of them has been tested in human subjects.

Alternatively, it has been hypothesized that rapid regeneration of endothelial cells without replication of VSMC may also modulate vascular growth because multiple antiproliferative endothelium-derived substances (prostacyclin 2, nitric oxide, C-type natriuretic peptide) are secreted from endothelial cells (see Fig. 1). This concept was first tested by over-expression of the $VEGF_{165}$ gene (Asahara et al., 1995). Asahara et al. (1995) reported a significant inhibition

Table 3
Potential targets for gene therapy in cardiovascular disease using decoy strategy

Gene therapy	Target transcription factor
Restenosis after angioplasty	E2F (Morishita et al., 1995), NF-κB
Hypertension	Angiotensinogen (Morishita et al., 1996)
Transplant vasculopathy	E2F, NF-κB
Myocardial reperfusion injury	NF-κB (Morishita et al., 1997; Sawa et al., 1997)
Inflammation	NF-κB
Graft failure	E2F (Mann et al., 1999), NF-κB

Modified from Morishita et al. (1998a).

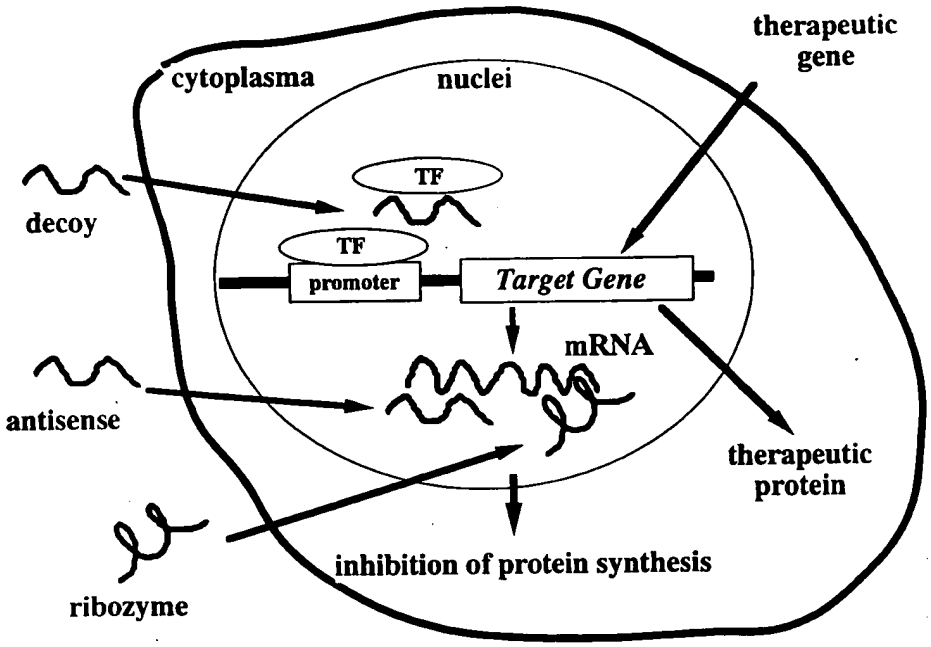

Fig. 1. Strategy for anti-restenosis.

of neointimal formation by acceleration of endothelial cell replication by VEGF gene transfer. Based on this finding, a human trial using the $VEGF_{165}$ gene by hydrogel catheter delivery of naked $VEGF_{165}$ plasmid DNA has been started for peripheral artery restenosis after angioplasty (Isner et al., 1996b). Although the final results have not been reported yet, the preliminary results documented the successful inhibition of restenosis after angioplasty (Vale et al., 1998). A similar trial using the $VEGF_{165}$ gene has been started in Finland. In this trial, the VEGF gene was transfected by cationic liposome or adenovirus with a catheter into the coronary artery (Laitinen et al., 2000). A recent report demonstrated the clinical safety of VEGF gene transfer with cationic liposome or adenovirus (Laitinen et al., 2000). In addition, we also reported preclinical experiments in which over-expression of the HGF gene in balloon-injured arteries could accelerate re-endothelialization, thereby attenuating intimal hyperplasia (Aoki et al., 2000). In this study, we also found that re-endothelialized balloon-injured arteries showed impairment of endothelial dysfunction (Aoki et al., 2000). Further studies are necessary to clarify the utility of gene therapy to treat restenosis after angioplasty.

3. Gene therapy for cardiovascular disease using oligonucleotide-based strategy

3.1. General concept

Recent progress in molecular biology has provided new techniques to inhibit target gene expression. Especially, the application of DNA technology such as antisense strategy to regulate the transcription of disease-related genes in vivo has important therapeutic potential. Antisense oligodeoxynucleotides (ODNs) are widely used as inhibitors of specific gene expression because they offer the exciting possibility of blocking the expression of a particular gene without affecting the function of other genes (see Fig. 2) (Ma et al., 2000). Therefore, antisense ODNs are useful tools in the study of gene function, and may be potential therapeutic agents. Another approach is the use of ribozymes, a unique class of RNA molecules that not only store information, but also possess catalytic activity (Kiehntopf et al., 1995). Ribozymes are known to catalytically cleave specific target mRNA molecules, leading to their degradation, whereas antisense ODNs inhibit mRNA translation by stoichiometrically binding to specific mRNA sequences. Theoretically, ribozymes are more effective inhibitors of target gene expression than are antisense ODNs. On the other hand, more recently, we have found a novel molecular strategy in which synthetic double-stranded DNA with high affinity for a target transcription factor may be introduced into target cells as a "decoy" cis-element to bind the transcription factor and to alter gene transcription (Morishita et al., 1998a).

3.2. Antisense or ribozyme-based gene therapy

As discussed in Section 2.2, angioplasty is limited by the development of restenosis in over 40% of patients (Gibbons & Dzau, 1994). Intimal hyperplasia develops in large part as a result of VSMC proliferation and migration induced by the complex interaction of multiple growth factors (Most &

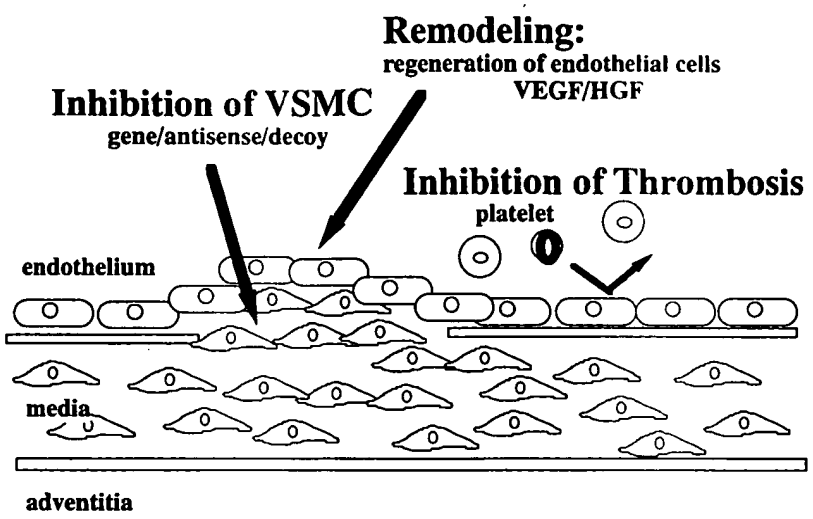

Fig. 2. Target sites for antisense, ribozyme, and decoy strategies. Antisense, antisense ODN; decoy, decoy ODN; ribozyme, ribozyme ODN; TF, transcription factor.

Sinnock, 1983). First, the effectiveness of antisense ODNs against a proto-oncogene, c-myb, was reported for the treatment of restenosis (Simons et al., 1992). Accordingly, inhibition of other proto-oncogenes such as c-myc by antisense ODNs was also reported to inhibit neointimal formation in several animal models (Shi et al., 1994). Currently, a Phase II trial using antisense c-myc to treat restenosis is underway, although its results have not been reported. However, as this trial utilized an intracoronary infusion of antisense c-myc ODNs, several issues such as low transfection efficiency may limit the efficacy of this strategy.

On the hand, the process of VSMC proliferation is dependent on the coordinated activation of a series of cell cycle regulatory genes that results in mitosis. Our previous data revealed that a single administration of antisense ODNs against proliferating-cell nuclear antigen (PCNA) and cdc 2 kinase genes inhibited neointimal formation after angioplasty at least up to 8 weeks after transfection (Morishita et al., 1993). In addition, a single administration of a combination of cyclin B_1/cdc 2 antisense ODNs significantly inhibited the extent of neointimal formation for a period of 8 weeks after transfection (Morishita et al., 1994a, 1994b). Similar trials have been employed for other vascular diseases, i.e., restenosis after vein grafting and vasculopathy in the transplanted heart. This proliferative vascular disease may also be an ideal target for an antisense ODN-based strategy. Mann et al. (1995) reported that transfection of antisense ODNs against PCNA and cdc 2 kinase resulted in the inhibition of hyperplasia 2 weeks after transfection in a vein graft model. Moreover, the prevention of neointimal formation by the cell cycle inhibition strategy, in the balloon injury model, was sustained long term over the period of antisense ODN survival (Morishita et al., 1993, 1994a, 1994b; Mann et al., 1995). This may relate to the vascular remodeling induced by the inhibition of cell cycle progression. Indeed, administration of antisense PCNA and cdc 2 kinase ODNs into a vein graft model improved the resistance to diet-induced atherogenesis (Mann et al., 1997). In addition to the prevention of restenosis after vein grafting, the inhibition of hyperplasia in transplanted hearts has also been reported by Suzuki et al. (1997). Transfection of antisense cdk2 kinase ODNs resulted in significant inhibition of VSMC growth in the transplanted heart (Suzuki et al., 1997). The first antisense drug appeared on the market in the United States at the end of 1999 as a novel drug to treat cytomegaloviral-mediated retinopathy.

Another strategy for combating disease processes is to target transcription via the use of ribozymes. We demonstrated the utility of ribozyme oligonucleotides against transforming growth factor-ß by targeting the common sequence of transforming growth factor-ß genes among human, rat, and mice to treat restenosis in a balloon injury carotid artery model (Yamamoto et al., 2000). In addition, we reported the inhibition of the production of lipoprotein (a), which is a risk factor for atherosclerosis, restenosis after angioplasty, cardiac disease, and stroke, without affecting the plasminogen level using a ribozyme strategy (Morishita et al., 1998b). Nevertheless, similar to the antisense ODN strategy, application of ribozyme technology to human gene therapy may require enhancement of the efficiency of cellular uptake and the stability of ribozyme oligonucleotides, since ribozymes are easily degraded by nucleases because of their RNA backbone.

3.3. Decoy-based gene therapy

Transfection of cells with decoys has been reported as a powerful tool in a new class of anti-gene strategies for gene therapy (Morishita et al., 1998a; Tomita et al., 1997). Transfection of cells with decoys will result in the attenuation of authentic cis-trans interactions, leading to the removal of trans-factors from the endogenous cis-element,

with subsequent modulation of gene expression (Fig. 2). Therefore, the decoy approach may also enable us to treat diseases by the modulation of endogenous transcriptional regulation. Currently, several studies have reported the application of the "decoy" ODN strategy for in vivo gene therapy (Morishita et al., 1995, 1996, 1997; Sawa et al., 1997). These studies provide evidence for the in vivo application of this novel molecular approach as a therapeutic strategy against cardiovascular disease. Many researchers employed antisense technology as a "loss of function" approach at the transcriptional and translational level, whereas the cis-element decoy strategy is also applicable as a "loss of function" approach at pre-transcriptional and transcriptional levels to study transcription factors.

As discussed in Section 2.2, the process of VSMC proliferation is dependent on the coordinated activation of a series of cell cycle regulatory genes, which results in mitosis. A critical element of cell cycle progression regulation involves the formation of the E2F-cyclin A/cdk 2 complex. The dissociation of the transcription factor E2F from the retinoblastoma gene product is proposed to play a pivotal role in the regulation of cell proliferation by inducing a coordinated transactivation of genes involved in cell cycle regulation, including c-*myc*, c-*myb*, cdc 2, PCNA, and thymidine kinase. Accordingly, we hypothesized that transfection of a sufficient quantity of decoy ODN containing the E2F cis-element (consensus sequence "TTTTCGGCGC") into VSMC would effectively bind E2F, prevent it from transactivating the expression of essential cell cycle regulatory proteins, and thereby inhibit VSMC proliferation and neointimal formation. Transfection of E2F decoy ODN into rat balloon-injured carotid arteries resulted in almost complete inhibition of neointimal formation 2 weeks after balloon injury (Morishita et al., 1995). Of importance, sustained inhibition of neointimal formation by a single administration of E2F decoy ODNs was observed at least up to 8 weeks after treatment. Inhibition of neointimal formation by E2F decoy delivered by hydrogel catheter was also demonstrated using a porcine coronary artery model. Delivery of ODNs by a hydrogel catheter may overcome issues such as the low transfection efficiency observed in coronary infusion. Based on these results, in April 2000, we started a clinical trial using a hydrogel catheter to deliver an E2F decoy to treat restenosis after angioplasty. As of March 2001, we have treated five patients with E2F decoy ODNs. We did not observe any side effects at up to 3 months, although the clinical outcome has not been evaluated yet.

In addition, in 1996, clinical application of a "decoy" against E2F by V. J. Dzau at Harvard University (Cambridge, MA, USA) was also approved by the United States Food and Drug Administration to treat neointimal hyperplasia in vein bypass grafts, which results in failure in up to 50% of grafts within a 10-year period. Recently, they demonstrated successful inhibition of graft occlusion, accompanied by selective inhibition of PCNA and c-myc expression (Mann et al., 1999). Since E2F has been postulated to play an important role in the pathogenesis of numerous diseases, e.g., glomerulonephritis and arthritis, the development of an E2F decoy strategy may provide a useful therapeutic tool for treating these proliferative diseases.

On the other hand, the transcription factor nuclear factor-κB (NF-κB) also plays a pivotal role in the coordinated transactivation of cytokine and adhesion molecule genes whose activation has been postulated to be involved in numerous diseases such as myocardial infarction. These diseases are extremely difficult to treat due to the lack of effective pharmacological agents, but are potentially amenable to ODN-based gene therapy. In addition to cell cycle regulatory genes, numerous cytokines, including interleukin-1, -2, -6, and -8 and tumor necrosis factor-α, to name a few, regulate the process of restenosis after angioplasty. Fortunately, gene regulation of many cytokines is relatively simple because the transcription factor NF-κB has been reported to up-regulate these cytokines. Interestingly, adhesion molecules, such as vascular cell adhesion molecule and intercellular adhesion molecule, are also known to be up-regulated by NF-κB. Accordingly, we hypothesize that restenosis could be prevented by the blockade of genes regulating cell inflammation — the final common pathway that is induced by NF-κB binding. The necessity to block cytokine and adhesion molecule genes at more than one point in order to achieve maximum inhibitory effects may be due to the redundancy and complexity of the interactions of these genes.

Importantly, increased NF-κB binding activity has been confirmed in balloon-injured blood vessels (Lindner, 1998). Recently, our preliminary study provided the first evidence of the feasibility of the decoy strategy against NF-κB in treating restenosis (Gibson, 1996). Transfection of NF-κB decoy ODNs into balloon-injured carotid artery or porcine coronary artery markedly reduced neointimal formation, whereas no difference was observed between scrambled decoy ODN-treated and untransfected rats. Based upon the therapeutic efficacy of this strategy, we will soon start a second clinical trial using the decoy strategy to treat restenosis. The blockade of NF-κB is also effective in the treatment of reperfusion myocardial injury (Most & Sinnock, 1983; Tyrrell & Wolfe, 1993; Sawa et al., 1997). Transfection of NF-κB decoy ODNs into the rat left anterior descending coronary artery prior to occlusion markedly reduced the area of damaged myocytes 24 hr after reperfusion. The therapeutic efficacy of this strategy via intracoronary administration immediately after reperfusion, similar to the clinical situation, was also examined. NF-κB decoy ODNs reduced reperfusioninduced injury to myocytes when compared with rats treated with scrambled control decoy or vehicle. In addition to the treatment of cardiovascular disease, NF-κB decoy ODNs are also effective in the treatment of glomerulonephritis and arthritis (Tomita et al., 1999; Tomita et al., 2000).

Since an important concern regarding the decoy strategy revolves around the potential inhibition of normal physiological responses, the application of the decoy strategy as gene therapy may be limited to the treatment of acute conditions; namely, "transcription factor-driven diseases." The decoy approach is particularly attractive for several reasons: (1) the potential drug targets (transcription factors) are plentiful and readily identifiable; (2) the synthesis of sequence-specific decoys is relatively simple and can be targeted to specific tissues; (3) knowledge of the exact molecular structure of the targeted transcription factor is unnecessary; and (4) decoy ODNs may be more effective than antisense ODNs in blocking constitutively expressed factors, as well as multiple transcription factors that bind to the same cis-element. Thus, the "decoy" strategy may be useful for treating a broad range of cardiovascular diseases (see Table 3).

3.4. Unresolved issues in oligodeoxynucleotide-based gene therapy

ODN-based gene therapy still has many unsolved problems, such as a short half-life, low efficiency of uptake, and degradation by endocytosis and nucleases. Therefore, currently, many groups are focusing on modifications of the gel approach using a catheter delivery system. Further modification of ODN pharmacokinetics will facilitate the potential clinical utility of the agents by (1) allowing a shorter intraluminal incubation time to preserve organ perfusion, (2) prolonging the duration of biological action, and (3) enhancing efficacy such that the nonspecific effects of high doses of ODNs can be avoided.

Regarding the ODN-based strategy as gene therapy, one of the major concerns is a nonspecific effect, particularly those of phosphorothioate-substituted ODNs. This concern is not only related to the antisense and decoy strategies, but also to all ODN-mediated therapies. Non-sequence-specific inhibition may operate through blockade of cell surface receptor activity or interference with other proteins (Gibson, 1996). At the same time, ODNs containing GC dinucleotides may bring about immune activation (Khaled et al., 1996). In addition, sequence-specific binding of nontranscriptional factor proteins to ODNs has been reported to result in nonspecific effects of ODN-based gene therapy (Henry et al., 1997a, 1997b). Moreover, Burgess et al. (1995) reported that the antiproliferative activity of c-myb and c-myc antisense ODNs in VSMCs is caused by a non-antisense mechanism. To overcome these issues, careful controlled experiments must be performed in order to eliminate the potential nonspecific effects of ODN-mediated therapy. For gene therapy using an ODN-based strategy, the toxicity of phosphorothioate ODNs may also be important. Although low-dosage administration does not seem to cause any toxicity, bolus infusions may be dangerous. Higher doses over prolonged periods of time may cause kidney damage, as evidenced by protein and leukocytes in the urine of animals (Henry et al., 1997a). Liver enzymes may also be increased in animals treated with moderate to high doses. Several phosphorothioate ODNs have been shown to cause acute hypotensive events in monkeys (Srinivasan & Iversen, 1995; Cornish et al., 1993), probably due to complement activation (Henry et al., 1997b). These effects are transient, if managed appropriately, and relatively uncommon. This toxicity can be avoided by intravenous infusions rather than bolus injections. More recently, prolongation of prothrombin, partial thromboplastin, and bleeding times has been reported in monkeys (Crooke, 1995).

4. Perspectives in gene therapy

Gene therapy in the field of cardiovascular disease would be useful for the treatment of many diseases, including PAD, myocardial infarction, restenosis after angioplasty, and rejection in heart transplantation. The first federally approved human gene therapy protocol started on September 14, 1990, for adenosine deaminase-deficient patients. Ten years since the commencement of the first trial, over 4000 patients have been treated by gene therapy. The objectives are generally to evaluate (1) the in vivo efficacy of the gene transfer method, (2) the safety of the gene transfer method, and (3) the possible therapeutic efficacy. Although there are still many unresolved issues in the clinical application of gene therapy, gene therapy for cardiovascular disease now appears to be not far from reality, and it is time to take a hard look at practical issues that will determine the real clinical potential. These include (1) further innovations in gene transfer methods, (2) well-defined disease targets, (3) cell-specific targeting strategies, and (4) effective and safe delivery systems.

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